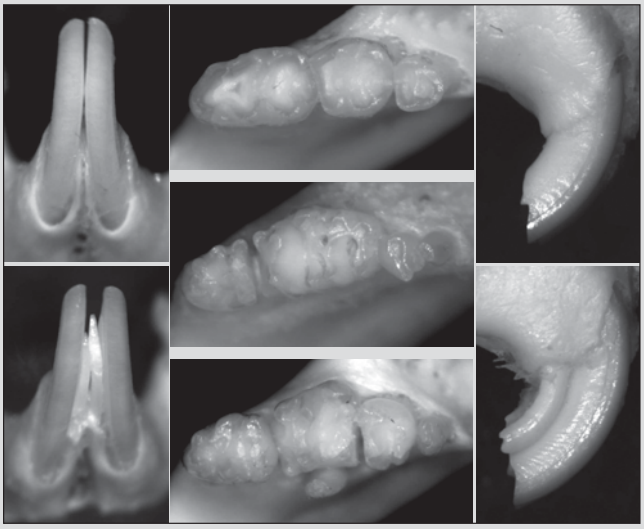

Sostdc1 Plays an Essential Role in Mammalian Tooth Patterning: Insight into the Rodent Dental Evolution

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles (referred in the text by their Roman numerals) and some unpublished results.

I Kassai, Y.*, Munne, P.*, Hotta, Y., Penttilä, E., Kavanagh, K., Ohbayashi, N., Takada, S.,Thesleff, I., Jernvall, J., Itoh, N. (2005).
Regulation of mammalian tooth cusp patterning by ectodin.
Science 309, 2067-2070.
* These authors contributed equally to this work

II Munne, P.M., Tummers, M., Järvinen, E., Thesleff, I., Jernvall, J. (2009).
Tinkering with the inductive mesenchyme: Sostdc1/Ectodin uncovers the role of dental mesenchyme in limiting tooth induction.
Development 136 (3), 393-402.

III Munne, P.M., Felszeghy, S., Jussila, M., Suomalainen, M., Thesleff, I., Jernvall, J. (2010).
Splitting placodes: effects of bone morphogenetic protein and Activinβ on the patterning and identity of mouse incisors.
Evolution & Development 12, 383-392.

ABBREVIATIONS

AER	apical ectodermal ridge
APC	Adenomatous polyposis coli
BMP	Bone morphogenetic protein
BMPR	Bone morphogenetic protein receptor
BrdU	Bromodeoxyuridine, synthetic nucleoside. Incorporates in proliferating cells.
BSA	bovine serum albumin
CaM1	Calmodulin1
CNC	cranial neural crest
Dkk	Dickkopf
DNA	deoxyribonucleic acid
Dpp	Decapentaplegic, BMP homologue in *Drosophila*
E	embryonic day
Eda	Ectodysplasin
Edar	Ectodysplasin receptor
FGF	Fibroblast growth factor
FSKO	Follistatin knock-out mouse
Fz	Frizzled Wnt-receptor
GFP	green fluorescent protein
Hh	Hedgehog
Homologous structure	Structure derived from a common ancestor. Function can be different.
Hox genes	Homeodomain containing transcription factors.
K	keratin
KO	knockout
Lef1/TCF	lymphoid enhancer factor1/ T-cell factor
LRP	low-density receptor-related protein family Wnt co-receptor
mRNA	messenger RNA
Msx	vertebrate homologue of *Drosophila* muscle segment (Msh) gene
Osr2	odd-skipped related2
p21	cyclin-dependent kinase inhibitor
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pax	Paired box containing transcription factor
PFA	paraformaldehyde
Pitx	Paired-like homeodomain
Ptc1	Patched1, Shh-receptor
RACS	replication-competent avian retroviruses
Sostdc1	Sclerostin domain containing 1
^{35}S-rUTP	Uridine 5'[α-^{35}S]thiotriphosphate
Shh	Sonic Hedgehog
Smad	vertebrate homologue of *Drosophila* mothers against decapentaplegic (MAD) gene
Smo	Smoothened, Shh signaling activator
Spry	Sprouty
TGF-β	Transforming growth factor beta
TNF	Tumour necrosis factor
Transplantation	Moving organ/tissue from one body part to other location.
Wnt	vertebrate homologue of the *Drosophila* Wingless gene

In the text gene names are written in italics and proteins in roman.

SUMMARY

This thesis work focuses on the role of TGF-beta family antagonists during the development of mouse dentition. Teeth develop through an interaction between the dental epithelium and underlying neural crest derived mesenchyme. The reciprocal signaling between these tissues is mediated by soluble signaling molecules and the balance between activatory and inhibitory signals appears to be essential for pattern formation. We showed the importance of Sostdc1 in the regulation of tooth shape and number. The absence of *Sostdc1* altered the molar cusp patterning and led to supernumerary tooth formation both in the molar and incisor region. We showed that initially, *Sostdc1* expression is in the mesenchyme, suggesting that dental mesenchyme may limit supernumerary tooth induction. We tested this in wild-type incisors by minimizing the amount of mesenchymal tissue surrounding the incisor tooth germs prior to culture *in vitro*. The cultured teeth phenocopied the extra incisor phenotype of the *Sostdc1*-deficient mice. Furthermore, we showed that minimizing the amount of dental mesenchyme in cultured *Sostdc1*-deficient incisors caused the formation of additional *de novo* incisors that resembled the successional incisor development resulting from activated Wnt signaling. Sostdc1 seemed to be able to inhibit both mesenchymal BMP4 and epithelial canonical Wnt signaling, which thus allows Sostdc1 to restrict the enamel knot size and regulate the tooth shape and number. Our work emphasizes the dual role for the tooth mesenchyme as a suppressor as well as an activator during tooth development. We found that the placode, forming the thick mouse incisor, is prone to disintegration during initiation of tooth development. The balance between two mesenchymal TGF-beta family signals, BMP4 and ActivinβA is essential in this regulation. The inhibition of BMP4 or increase in ActivinβA signaling led to the splitting of the large incisor placode into two smaller placodes resulting in thin incisors. These two signals appeared to have different effects on tooth epithelium and the analysis of the double null mutant mice lacking *Sostdc1* and *Follistatin* indicated that these TGF-beta inhibitors regulate the mutual balance of BMP and Activin *in vivo*. In addition, this work provides an alternative explanation for the issue of incisor identity published in Science by Tucker et al. in 1998 and proposes that the molar like morphology that can be obtained by inhibiting BMP signaling is due to partial splitting of the incisor placodes and not due to change in tooth identity from the incisor to the molar.

This thesis work presents possible molecular mechanisms that may have modified the mouse dental pattern during evolution leading to the typical rodent dentition of modern mouse. The rodent dentition is specialized for gnawing and consists of two large continuously growing incisors and toothless diastema region separating the molars and incisors. The ancestors of rodents had a higher number of more slender incisors together with canines and premolars. Additionally, murine rodents, which include the mouse, have lost their ability for tooth replacement. This work has revealed that the inhibitory molecules appear to play a role in the tooth number suppression by delineating the spatial and temporal action of the inductive signals. The results suggest that Sostdc1 plays an essential role in several stages of tooth development through the regulation of both the BMP and Wnt pathway. The work shows a dormant sequential tooth forming potential present in wild type mouse incisor region and gives a new perspective on tooth suppression by dental mesenchyme. It reveals as well a novel mechanism to create a large mouse incisor through the regulation of mesenchymal balance between inductive and inhibitory signals.

“The vertebrate dentition is an evolutionary enigma. It is a critical organ system for survival, and yet it is among the most variable characters in vertebrate history.” – Carroll, 1988

1. INTRODUCTION

One of the classical examples of the environmental influence on facial morphology is Darwin's finches (*Geospiza*) in the Galapagos Islands. The selection pressure has favored the specialization of the finches to different diets and led to the development of diverse beak shapes. Modularity of the organism allows this adaptive phenotypic evolution. The different structures are thus able to change semi-autonomously. In vertebrates the jaw, beak, and skull form the craniofacial skeleton, which can be highly variable among closely related species. Therefore one way to explore the molecular bases for different craniofacial phenotypes is to investigate differences in the gene expression patterns between two closely related species that represent differences in their craniofacial appearance (**Abzhanov et al. 2004**). As the phenotypic diversification can take place considerably fast and the changes often occur in a highly conserved genetic context, the phenotypic variation is often considered to take place through modifications of the underlying genetic network rather than recruiting new genes. The regulation of genes may take place at many different levels such as involving changes in the gene regulatory elements, in the micro-RNA levels or in the expression patterns of their antagonistic molecules.

Studies of the fossil record have documented the variation in the mammalian tooth morphology throughout the evolutionary history. Changes in the developmental processes are considered to underlie evolutionary changes by producing new features in the evolving lineages (**Kim et al. 2000; Raff, 2000**). The increasing molecular evidence has started to give insight into those developmental processes, which may be involved in the modulation of tooth shapes between two different species (**Keränen et al. 1998**). The increasing knowledge of the molecular mechanisms of morphogenesis has complicated the separation between structure and function (**Gilbert and Bolker, 2001**). Several genes appear to assemble in functional modules that can be adapted to the development of different structures. The use of the same genes in the similar processes in different organs does not, however, imply the homology of different structures, but indicates the conservation of developmental mechanisms and how widely they are used during development (**Gilbert and Bolker, 2001**). Therefore a mutation in one gene usually causes simultaneous changes in many different structures. In addition to this evolutionary aspect, there is also a medical interest in understanding craniofacial formation since the malformations in the craniofacial development has been reported as the major cause for human congenital defects (**Chai and Maxson, 2006**). In order to understand the pathophysiology behind these events it is crucial to understand first the normal developmental processes. Genetically modified animals serve as an excellent model to investigate the genetic network behind normal development as well as the molecular bases for different craniofacial phenotypes.

2. REVIEW OF THE LITERATURE

2.1. Neural crest

The formation of neural crest cells and their recruitment to craniofacial development is a novelty of vertebrate head development. Neural crest cells arise from the dorsal edges of the folded neural tube. These cells migrate and differentiate into many different kinds of cell types such as melanocytes, sensory neurons, and Schwann cells. The neural crest cells that originate from the cranial region have additional ability to form skeletogenic tissue. These cranial neural crest cells form most of the craniofacial skeleton in the vertebrates. They delaminate from the neuroectoderm and migrate ventrally to colonize the facial processes and pharyngeal arches. These cells seem not to express *Hox*-genes, homeodomain containing transcription factors, to pre-pattern the facial structures. In the pharyngeal arch these cells form skeletogenic condensations and differentiate to form facial and cranial structures. The contribution of neural crest cells to tooth and jaw development was shown with the two component genetic system, where Cre-recombinase was expressed under the Wnt1-promoter as a transgene in one mouse line and the recombinase activity was detected after crossing the mice with conditional reporter line ROSA26 (**Chai et al. 2000**). This allowed the researchers to follow the migration and differentiation of cranial neural crest cells. Wnt1 has been shown to be a neural crest cell marker (**Dorsky et al. 1998**). As a result, bones of the calvarium, face, jaws, and most structures of the teeth were observed to form from the cranial neural crest cells. The classic transplantation experiments in urodele amphibians showed that grafted neural crest cells from the trunk region failed to form skeletogenic tissue when placed at the cranial region (**Raven, 1931; Hörstadius and Sellman, 1945; Chibon, 1966**; **Graveson, 1993; Graveson et al. 1995**). The skeletogenic ability appears to be a feature only for the cranial neural crest cells and require interaction with the ecto- and endodermal tissues.

The neural crest cells are thought to carry the information needed for the construction of the cranial skeleton (**Noden, 1983; Tucker et al. 2004a; Merrill et al. 2008**). Bone morphogenetic protein4 (BMP4) appears to have an important role in the formation of skeletogenic precursors from cranial neural crest cells and their migration to the right location. The transgenic overexpression of the BMP antagonist, *Noggin*, in the premigratory/migratory neural crest cells in the cranial region led to the depletion of cranial neural crest cells, hypomorphic branchial arches, and the absence of their skeletal and neural derivatives (**Kanzler et al. 2000**). The conditional deletion of *Bmp4* from the mandibular ectoderm and branchial arch endoderm in transgenic mice led similarly to the complete loss of the mandible in mice (**Liu et al. 2005a**). In addition, canonical Wnt signaling seems to be important for craniofacial development. This was observed from the *Wnt1*-cre mediated depletion of β-catenin in neural crest cells, which led to the absence of the entire craniofacial skeleton (**Brault et al. 2001**).

2.2. Placodes and initiation of ectodermal organogenesis

The different ectodermal organs such as tooth, hair, scale, feather, beak or mammary gland share similarities in their early development. They start to form from non-neural ectoderm-derived structures called placodes and their further development is regulated by common

signaling pathways (**Pispa and Thesleff, 2003**). Placodes, like neural crest cells, are considered to be evolutionary novelties of vertebrates (**Shimeld and Holland, 2005**). They are the first visible signs of ectodermal organ development. The underlying mesenchyme condensates under the placode to form a structure called papilla (**Jiang et al. 1999**) and together with placode they form organ primordium. The relative contribution of cell proliferation and cell migration in the placode formation is still relatively poorly understood (**Balinsky, 1950; Wessells, 1965; Magerl et al. 2001**). Depending on the forming structure, the development of the placodes may vary a lot. They invaginate to the underlying mesenchyme in tooth and hair development and evaginate to form feathers. The genes involved for the placode formation and function are similar between the ectodermal organs. The lack of *p63* for instance arrests the ectodermal organ development prior the placode formation (**Laurikkala et al. 2006**). During tooth development the placodes form as local thickenings of the primary epithelial band called dental lamina (**Iseki et al. 1996; Pispa and Thesleff, 2003**). Each tooth family is thought to form successively from one primary tooth placode. Therefore in mouse these placodes form only at the site of the incisors and the first molars. The balance between activatory and inhibitory signals appears to affect the initiation decision in placode formation. The fine-tuning of these signals affects the future organ pattern. The reaction-diffusion process is thought to establish the pattern of organ primordia (**Jung et al. 1998; Widelitz et al. 2000**). The primordium cells start to secrete inhibitors to prevent the neighboring cells from developing the same fate. This causes a spatial variation between activator and inhibitor molecules and forms a zone of inhibition around the developing placode, which prevents the formation of a new primordium. Further growth allows the initiation of new primordia. Osborn 1971 observed this dynamic from the development of the dentition. The teeth seem to be separated from each other by an inhibition field. This field surrounds every developing tooth and prevents the neighboring teeth to develop too close. Sonic hedgehog (Shh) signaling appears to have important role in tooth placode formation. Sarkar et al. 2000 showed that the overexpression of *Wnt7b* in the mouse oral ectoderm was able to repress the placodal *Shh* expression and thus prevented the tooth formation.

2.3. Tooth development

Erupted teeth consist mainly of calcified tissues, including dentin, cementum (secreted by the root), and enamel. The mesenchyme will give rise to the dentin producing odontoblasts, pulp, and the alveolar bone, and the epithelium will contribute to the enamel secreting ameloblasts (**Thesleff and Hurmerinta, 1981**). Dentin is secreted by the neural crest cells derived odontoblasts (**Lumsden 1988**) and it consists of collagen, dentin, sialophosphoprotein, dentin matrix protein, and hydroxylapatite. The dentin is covered by a layer of enamel secreted by the epithelial derived ameloblasts. Enamel is practically collagen-free and forms the hardest tissue of the body. This, together with highly mineralized dentin explains the persistence of teeth in the fossil record and allows the tracing of the evolutionary history based on the dental morphology.

Tooth development has served as an excellent model system to investigate the mechanisms of patterning, morphogenesis, and cytodifferentiation. In laboratory settings, mouse tooth development can be genetically manipulated and teeth can be readily cultured and manipulated *in vitro*. The development can be divided into three phases: initiation,

morphogenesis, and cell differentiation (**Kollar and Lumsden, 1978**). The development is largely mediated by the reciprocal signaling between two tissues, epithelium and mesenchyme, which gradually attain higher level of differentiation. Transgenic mouse models and transplantation studies have shown that the organogenesis of many ectodermal organs starts from the mesenchymal signal, which determines the pattern and identity of the structure (**Cairns and Saunders, 1954; Sengel, 1964; Dhouailly, 1975; Mitsiadis et al. 2003; Foley et al. 2001; Veltmaat et al. 2003**). Tooth development is, however, different. During the initiation, the odontogenic potential and patterning information resides in the epithelium, which also determines the tooth type in tissue recombination experiments (**Mina and Kollar, 1987; Kollar and Mina, 1991; Miller, 1969; Lumsden, 1988).** The neural crest derived mesenchyme is, however, required to induce the odontogenic program. Classic tissue recombination experiments in mouse showed that the early mandibular arch epithelium (E9–E11) was able to induce tooth formation when recombined with neural crest derived mesenchyme from the trunk region. The recombination with non-neural crest derived mesenchyme was, however, inadequate for tooth formation (**Mina and Kollar,**

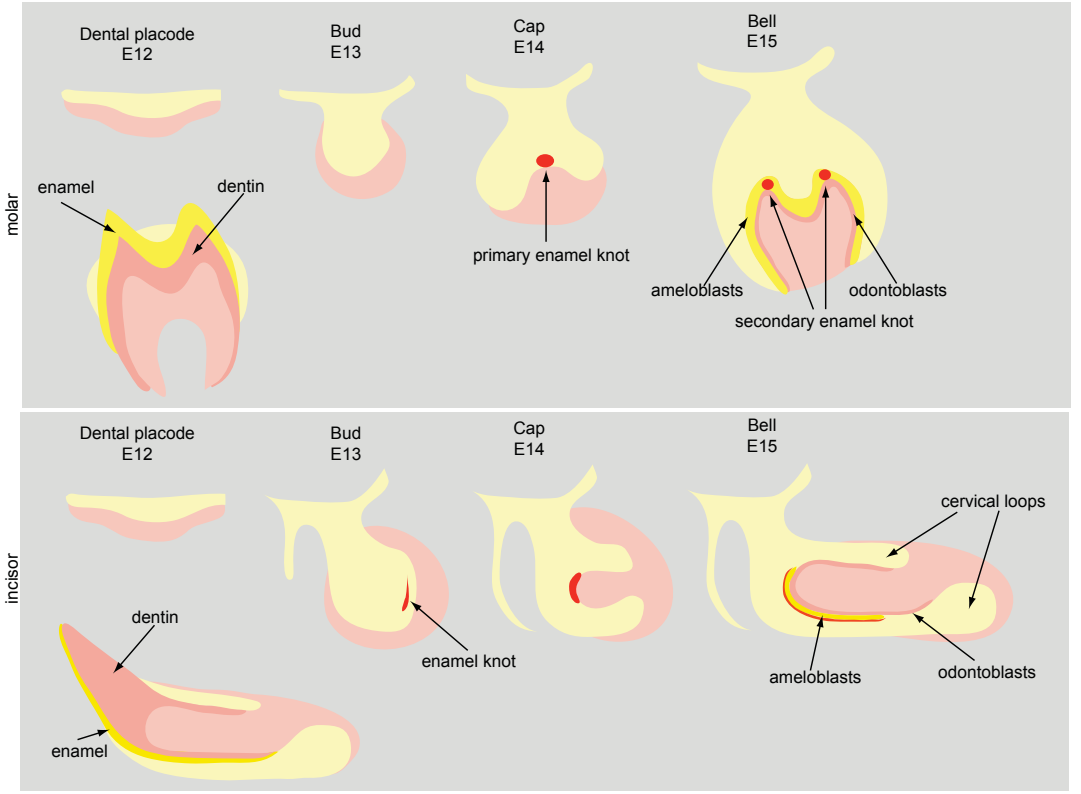


Figure 1. *Embryonic tooth development starts at E11, when the oral epithelium forms a thickening called the dental lamina. The tooth placodes appear at E12 and develop as buds at E13. The underlying mesenchyme condenses around these epithelial buds. At E14 the signaling center, the enamel knot, forms and regulates tooth morphogenesis. In the incisors the enamel knot forms a bit earlier, as the development is slightly advanced compared to the molars. Ameloblasts secrete enamel, whereas the odontoblasts form dentin. In the incisors, enamel forms only at the labial surface. Cervical loops contain the stem cell niche that allows the continuous growth of the rodent incisors.*

1987, Lumsden, 1988). The ability of mesenchyme to control the epithelial morphogenesis and maintenance of the tooth identity during the later tooth development was shown with tissue recombination studies (**Kollar and Baird, 1969; Gaunt and Miles, 1967**).

Results obtained from the heterospecific tissue recombination studies between mouse and chick showed that the lack of oral teeth in birds is considered to follow from the failure in the epithelial-mesenchymal interaction (**Kollar and Fisher, 1980**). The oral epithelium still retains the signaling properties needed for the initiation of an odontogenesis-like process, but the mesenchyme has lost odontogenic potential (**Kollar and Fisher, 1980; Chen et al. 2000**). The first sign of tooth formation in mice appears at embryonic day 11 (E11) as local thickenings of the oral epithelium, the dental lamina. Incisor initiation from the placode occurs about half day earlier than the molar. Tissue recombination studies have revealed that the potential to instruct tooth development shifts to the neural crest derived mesenchyme at E12 (**Kollar and Baird, 1970; Mina and Kollar, 1987**). *Bmp4* expression follows this change in the odontogenic potential (**Vainio et al. 1993**). In the bony fishes, the region-specific absence of teeth is correlated with the loss of mesenchymal *Bmp4* (**Wise and Stock, 2006**). In addition to *Bmp4* the early odontogenic mesenchyme expresses other important molecules that signal to the epithelium such as *Fibroblast growth factor3* (*Fgf3*), and *Activin*β*A*, and the canonical Wnt pathway **(Vainio et al. 1993; Ferguson et al. 1998; Kettunen et al. 2000; Chen et al. 2009**). At E12.5–E13.5 tooth development proceeds to bud stage through the epithelial proliferation and the mesenchyme cells condense around the invaginated epithelial tooth bud.

2.4. Enamel knot and tooth morphogenesis

The enamel knot is required for tooth development to proceed from the bud to cap stage. It is a site of non-proliferative epithelial cells, which affects the differentiation and proliferation of the surrounding epithelium and thus patterns the tooth shape by regulating the growth and folding of the inner dental epithelium (**Jernvall et al. 1994**). After the primary enamel knot the secondary enamel knots appear and regulate the formation of further tooth cusps. The unequal growth of the dental papilla also affects the shape and patterning of the forming tooth crown (**Butler, 1995**). Radioactive *in situ* hybridization studies have revealed that the enamel knot expresses more than 10 signals belonging to the conserved families, including *Shh*, several *Bmps*, *Fgfs*, and *Wnts* (**Jernvall et al. 1994; Vaahtokari et al. 1996a; Thesleff, 2003**). FGFs are known to stimulate epithelial and mesenchymal cell proliferation. This was observed by a BrdU cell proliferation assay after the introduction of recombinant protein beads to the tooth mesenchyme (**Kettunen et al. 1998**). The signals expressed by the enamel knot are also found in other embryonic signaling centers, such as the apical ectodermal ridge (AER) in the limb development.

Mathematical modeling has been used to discover the dynamics of molecular interactions during pattern formation (**Salazar-Ciudad and Jernvall, 2002, 2010**). According to the mathematical model, the tooth shape results from the dynamic use of an activator-inhibitor loop (**Salazar-Ciudad and Jernvall, 2002, 2010**). This mathematical model, together with experimentation, has implicated BMP4 as an activator in tooth patterning (**Jernvall et al. 1998**; **Salazar-Ciudad and Jernvall, 2002; Kassai et al. 2005; Kavanagh et al. 2007).** Mesenchymal BMP4 is needed for enamel knot activation and mice lacking the mesenchymal expression of transcription factor *Msx1*, a downstream

Table 1. *Selected genes which arrest tooth development before enamel knot formation when deleted or overexpressed.*

Mutants	Mandibular phenotype	References
Msx1 -/-	Tooth arrest at the lamina stage.	Chen et al. 1996.
Msx1/2 -/-	Tooth arrest at the lamina stage.	Bei and Maas, 1998.
p63 -/-	Tooth arrest at the lamina stage.	Laurikkala et al. 2006.
Pitx2 -/-	Tooth arrest at the placode	Lin et al. 1999.
Fgf8 -/-	Loss of molars.	Trumpp et al. 1999.
Fgfr2b -/-	Tooth arrest at the bud stage.	De Moerlooze et al. 2000.
Gli2/3 -/-	Tooth arrest at the bud stage.	Hardcastle et al. 1998.
Lef1 -/-	Tooth arrest at the bud stage.	van Genderen et al. 1994.
Pax9 -/-	Tooth arrest at the bud stage.	Peters et al. 1998.
Runx2 -/-	Tooth arrest at the bud stage.	D'Souza et al. 1999.
ActivinβA -/-	Tooth arrest at the bud stage.	Matzuk et al. 1995.
Bmpr1a -/-	Tooth arrest at the bud stage.	Andl et al. 2004.
Prx1/2 -/-	Tooth arrest at the bud stage.	ten Berge et al. 1998.
K14-Dkk1	Tooth arrest at the bud stage.	Andl et al. 2002; Liu et al. 2008.

target of BMP4, fail to form the enamel knot and tooth development is arrested at the bud stage (**Chen et al. 1996**). The enamel knot also fails to form in mice lacking epithelial *Bone morphogenetic protein receptor IA* (*BmprIA*) expression (**Andl et al. 2004**). The stabilization of epithelial β-catenin was observed to be absent in mice that have a conditional deletion in their *Bmpr1A* gene. The BMPR-IA activity was thus positioned genetically upstream of epithelial β-catenin stabilization (**Andl et al. 2004; Soshnikova et al. 2003).** The introduction of recombinant BMP4 protein to the tooth epithelium induced the expression of the first differentiation marker, cyclin-dependent kinase inhibitor *p21*, in the enamel knot (**Jernvall et al. 1998**). Enamel knots are transient epithelial structures and the epithelial expression of *Bmp4* in the enamel knot has been suggested to terminate the enamel knot and cause its apoptotic disappearance (**Vaahtokari et al. 1996a; Jernvall et al. 1998**). The epithelial *Bmp4* expression has been shown to be dependent on *Msx2*, which is induced by *Bmp4* itself (**Bei et al. 2004**). In the *Msx2* loss-of-function mice, molar cusp morphogenesis and proper amelogenesis were affected (**Bei et al. 2004**). Based on the observations from the BrdU assay, the Msx2 appeared to control the rate of cell proliferation in the enamel knots. In addition, the Msx2 was observed to be required for the expression of the extracellular matrix component, *Laminin 5 alpha 3*, which is important for ameloblast differentiation.

2.5. Morphogens and pattern formation

Mathematical model has been used to describe the formation of pigmentation patterns in animals, as well as the distribution of the epithelial appendages (**Turing, 1952**). This model is also suitable to explain the development of the segmented structures such as teeth, vertebrae, phalanges, and feathers and how they are lost during the evolution inversely to their appearance. These kinds of models have been applied to explain the patterning of the

molar tooth cusps and feathers (**Salazar-Ciudad and Jernvall, 2002, 2010**; **Michon et al. 2008**). These models are based on the reaction-diffusion dynamics and thus include secreted activator and inhibitor molecules and their reciprocal signaling loops, which pattern the embryo during the development. These molecules diffuse from the same source location and form a morphogen gradient that affects the fate of the surrounding cells (**Turing 1952**; **Wolpert 1969**). The cells differentiate according to their location in this morphogenetic field. The different concentrations create different responses in the gene expression of the recipient cells. The induction of different genes in the gradient depends on the threshold concentration. Lewis Wolpert used the French flag to describe the morphogen: the high concentration activates "the blue gene", lower concentration activates "the white gene", and the red represents the gene below the threshold concentration. The known morphogens seem to be effective in very low concentrations, but the rate of spread and the stability of the protein is still not known. Most morphogens are protein ligands that bind transmembrane receptors to initiate a cellular signaling pathway and the transcription of specific genes. The changes in the balance of the molecular activities cause morphoregulation. Members of the *Transforming growth factor beta*- (*Tgf*-β–), *Hedgehog*- (*Hh*-), and *Wnt*-families were shown to form morphogen gradients (**Gurdon and Bourillot, 2001**). In vertebrate morphogenesis ActivinβA, BMP, and Shh form gradients. The injection of different levels of *Decapentaplegic*, a member of TGF-beta family member, to the early *Drosophila* embryo caused the formation of more dorsal structures at high levels compared to low levels, which promoted the ventral fate. In the amphibian embryo, another TGF-beta family member, Activin, was shown to affect the genes expressed by a single cell depending on the cell's distance from the Activin source location. Even slight changes in the levels of morphogens are sufficient to switch cells between alternative fates (**Ferguson and Anderson, 1992; Gurdon et al. 1994**). Mutations in the genes that encode morphogens often cause severe phenotypes. Even hypomorphic mutations in these genes may alter the phenotype (**Dunn et al. 1997**). This indicates their key role in development. Morphogen gradients can be actively regulated by inhibitor molecules (**Jones and Smith, 1998**). The BMP4 gradient for example was shown to be established by inhibitor molecules such as Noggin and Chordin instead of BMP4 diffusion. The complete absence of one particular inhibitor molecule may thus cause a strong phenotype and even a hypomorphic mutation may be sufficient to perturb the phenotype. During hair development, Wnts are postulated to be activator molecules and Dickkopf1 (Dkk1) functions as an inhibitor (**Sick et al. 2006**). Both are expressed in the epithelial compartment of the skin. An increase in the level of activator molecule decreases the spacing between hair follicles and disrupts the pattern. An increase of the inhibitor, on the other hand, increases the interfollicular spaces.

2.6. Signaling pathways in tooth development

2.6.1 Fibroblast growth factor (FGF)

The fibroblast growth factor (FGF) family constitutes at least 22 members (**Itoh and Orniz, 2008**). They are widely expressed during embryonic development and regulate epithelial-mesenchymal interactions in multiple organs and tissues. According to BrdU studies, FGFs seem to promote cell proliferation both in the dental epithelium and mesenchyme during tooth development (**Jernvall et al. 1994; Kettunen and Thesleff, 1998**). *Fgf8* and *Fgf9*

are expressed in the early odontogenic epithelium at the time of tooth initiation (E10). Protein bead experiment have shown that they are both able to induce *Msx1* expression in the mandibular arch mesenchyme (**Kettunen et al. 1998**). Cre-mediated deletion of *Fgf8* in the branchial arch epithelium prevents the development of most of the first branchial arch derivatives, such as skeletal elements of the jaw, middle ear, and molar teeth to form, except for elements of distalmost region, such as the lower incisors (**Trumpp et al. 1999**). *Fgf4* and *Fgf9* are expressed in the primary and secondary enamel knots, the signaling centers of the developing tooth (**Jernvall et al. 1994; Kettunen and Thesleff, 1998**). They are expressed in the non-dividing cells, but they seem to increase the proliferation of the surrounding epithelial and mesenchymal tissues. *Fgf4* is considered as a direct target of the transcription factor Lef1 in the developing tooth, since the recombinant FGF4 protein was able to overcome the developmental arrest of *Lef1*-deficient-tooth (**Kratochwil et al. 2002**). The mesenchymal Wnt/β-catenin activity appears to contribute to the epithelial *Fgf4* expression (**Chen et al. 2009**). FGF10 has been shown to be critical for the survival of the epithelial stem cell niche in the mouse incisor tooth germ (**Harada et al. 2002**). It is expressed in the dental mesenchyme together with *Fgf3* at the cap and bell stages (**Wilkinson et al 1989; Harada et al. 2002**). FGF10 enables the renewal of dental epithelium by maintaining the epithelial stem cell population in mouse incisor cervical loop. Transgenic mice and bead experiments have shown FGF3 to participate in the enamel formation and to co-operate with FGF10 to keep the epithelial cells proliferating (**Wang et al. 2007**). *Sprouty* genes are intracellular inhibitors of FGF signaling and are necessary to restrict FGF signals during tooth development (**Klein et al. 2006**). FGF inhibition at the lingual side of the continuously growing mouse incisors prevents enamel production by the ameloblasts (**Klein et al. 2008**). The simultaneous absence of both *Sprouty2* and *Sprouty4* led to the formation of enlarged incisors with enamel present on both sides of the tooth (**Klein et al. 2008**). In the *Sprouty2*-deficient mouse, a vestigial diastema tooth was observed to appear anterior to the first molar and a slight change in the molar tooth crown was observed.

2.6.2 Sonic hedgehog (Shh)

Of the three hedgehog genes, only *Sonic hedgehog* (*Shh*) expression has been localized in a tooth (**Bitgood and McMahon, 1995**). It is restricted to epithelium and is localized in the placode, enamel knots, and differentiating ameloblasts. Shh has been shown to regulate tooth growth, morphogenesis, and cell polarization. It is considered as a key regulator of vertebrate tooth induction since it stimulates epithelial cell proliferation to form a tooth bud (**Bitgood and McMahon, 1995; Hardcastle et al. 1998; Cobourne et al. 2001**). This was based on studies of *Shh* expression patterns and the introduction of Shh releasing beads on dental epithelium. At the cap stage *Shh* is expressed in the signaling center, the enamel knot, from where it spreads to the inner enamel epithelium at the later stages (**Dassule et al. 2000**; **Koyama et al. 1996; Vaahtokari et al. 1996a**). Reduction and then loss of *Shh* at the bud stage prevents the tooth development to proceed beyond the cap stage (**Dassule et al. 2000**). The morphology of the tooth is severely disrupted although the enamel knot is able to form. The tooth is smaller and the lingual cervical loop is absent. The tooth is capable of producing enamel and dentin, but in disorganized layers (**Dassule et al. 2000**). The factors that induce *Shh* expression in the dental epithelium are currently unknown. BMP4 seems, however, to be involved in *Shh* expression (**Zhang et al. 2000**). The inhibition of BMP4 by

a Noggin releasing bead repressed the epithelial expression of *Shh* and *Bmp2* in the wild type tooth epithelium. Similarly the introduction of a BMP releasing bead in the *Msx1*-deficient tooth mesenchyme was able to restore the epithelial *Shh* and *Bmp2* expression. The introduction of BMP releasing bead on the wild type tooth epithelium led, however, to the down regulation of *Shh* in the tooth epithelium.

2.6.3 Ectodysplasin (EDA)

Ectodysplasin (EDA) is a member of tumor necrosis factor (TNF) signal family. It is the first member of this family, which has been implicated in ectodermal organ development (**Headon and Overbeek, 1999; Mikkola et al. 1999**). EDA is a secreted molecule that is expressed in a complementary manner to its receptor *Edar* in the tooth epithelium (**Laurikkala et al. 2001**). Cells expressing Wnt6 were able to induce *Eda* after being placed on a tooth epithelium (**Laurikkala et al. 2001**). ActivinA releasing beads were shown to induce *Edar* expression in tooth epithelium. EDA signaling appears to be essential for the development of all ectodermal organs, since the absence of this signaling cause hypohydrotic ectodermal dysplasia (HED), which has multiple defects in the hair, tooth, and gland formation. A microarray screen of the developing hair follicles revealed BMP inhibitors, *Shh*, and Wnt inhibitors, such as *Lrp4* and *Dkk4*, as downstream targets of EDA (**Fliniaux et al. 2008**). There is a naturally occurring mouse mutant for *Eda* called Tabby (**Falconer, 1952**). It has impaired development in multiple ectodermal organs, including reduced tooth size and number. The molars are smaller than normal and the third molars and incisors may occasionally be absent (**Grüneberg, 1965; Pispa et al. 1999**). The EDA pathway appears to be an important component in the evolution of ectodermal organs. In stickleback fishes, EDA regulates armor plate formation (**Colosimo et al. 2005**). In Medaka fish, the absence of *Edar* prevents scale formation (**Kondo et al. 2001)** and in zebrafish, both *Eda* and *Edar* mutations were observed to cause the lack of scales (**Harris et al. 2008**). Mice overexpressing *Eda* under the K14 promoter in the epithelium have supernumerary ectodermal organs such as an extra tooth in front of the first molar and the molars have aberrant cusp patterns (**Mustonen et al. 2003**). They also have impaired enamel in the incisors, which is consistent with the observation that EDA has been shown to inhibit *Bmp4* (**Pummila et al. 2007**). Ectopic expression of BMP antagonist, *Noggin*, under K14-promoter in the tooth epithelium, was shown to impair the enamel formation (**Plikus et al. 2005**). The overexpression of the EDA receptor, *Edar*, alters the cusp pattern, tooth number, and increases the mesenchymal *Bmp4* expression (**Pispa and Thesleff, 2003; Pispa et al. 2004**).

*2.6.4 Transforming growth factor-*β *(TGF-*β*) superfamily*

More than 30 members belong to the transforming growth factor-β (TGF-β) superfamily including Nodal, BMPs, TGF-βs, and Activins (**Reddi et al. 1994; Kawabata et al. 1998**). These soluble ligands bind to cell surface type-II and type-I serine/threonine kinase receptors. Five different type II receptors and seven different type I receptors have been identified in mammals **(Miyazono et al., 2000**). The type-II receptor transphosphorylates the type-I receptor, which leads to the phosphorylation of the cytoplasmic Smad-proteins.

Smad 1, 5, and 8 are reported to be responsive to BMP signals whereas Smad 2 and 3 seem to respond to ActivinβA and TGF-β signaling. Smad4 appears to be a common mediator for all TGF-β pathways. Smad6 and Smad7 interfere with the other Smads and are therefore considered as inhibitors of these pathways. During embryogenesis the TGF-β-family members are important for patterning and mesoderm formation. They are also needed in the regeneration of adult tissues as in wound healing, bone remodeling, and repair.

*2.6.4.1 Activin*β*A*

Like the signaling molecules in general, ActivinβA and its receptors are highly conserved throughout evolution from fishes to mammals suggesting their fundamental role in animal development (**Matzuk et al. 1996**). ActivinβA is a morphogen and it has been shown to form a passively diffusing gradient from its source location in an early embryo (**Gurdon et al. 1994)**. Cells express a different set of genes depending on the ActivinβA dose they are receiving. During tooth and hair follicle development ActivinβA has been shown to induce *Edar* expression in the epithelium (**Laurikkala et al. 2001**). Radioactive *in situ* hybridization has revealed *Activin*β*A* expression in the condensing mesenchyme around the tooth placodes already at E12 stage. During later development, recombinant ActivinβA protein has been shown to stimulate epithelial proliferation in the incisor cervical loop (**Wang et al. 2007**). Studies from *Activin*β*A*–deficient mice have shown that during the early mandibular development, ectodermal FGF8 is required for *Activin*β*A* expression in the tooth mesenchyme and that in the absence of *Activin*β*A*, incisors and mandibular molars fail to develop beyond the bud stage (**Ferguson et al. 1998)**.

2.6.4.2 Bone morphogenetic protein (BMP)

Bone morphogenetic proteins (BMPs) were discovered in the 1960's after injecting bone extract into the rabbit muscle leading to the ectopic bone formation (**Urist, 1965**). BMPs were found independently in Drosophila and named decapentaplegic (Dpp) (**Spencer et al. 1982**). BMPs have different roles in various cell types during development, such as apoptosis, growth, and differentiation. They are required for the development of most organs and tissues, including tooth and skeletal development, and limb morphogenesis. During embryonic development, BMP4 is important in mesoderm formation and *Bmp4*-deficient mice die early in development (E7.5–9.5) with defects in the mesoderm, allantois, and posterior structures (**Winnier et al. 1995**). BMP signaling is regulated through many different antagonists. These inhibitors seem to have an important role in multiple processes such as dorso-ventral patterning, induction of neural tissue, and formation of joints in the skeletal system. The regulation of BMPs by their antagonists takes place at many different levels (for review, **Miyazono, 2000**). Firstly, there are many extracellular antagonists (e.g., Noggin, Follistatin, Sostdc1, Chordin, Gremlin1, Cerberus, DAN) that can bind BMP ligands and inhibit their interaction with BMP receptors. In addition, there is a transmembrane inhibitor, Bambi, and intracellular inhibitors such as Smad6, which can prevent the phosphorylation of Smad1 and -5 by preventing them from binding BMP receptors. Some other molecules, such as an anti-proliferative protein Tob and a Smad ubiquitin regulatory factor 1, Smurf1, are able to interfere with Smad1 and -5 signaling

intracellularly by binding to them and preventing their signal transduction. Inside of a nucleus the Bmp-Smad signaling is regulated by proteins e.g. Ski. Tob is also mainly located in the nucleus (**Kawamura-Tsuzuku et al. 2004**).

BMP signaling is able to create a negative feedback loop to control its own activity by inducing expression of its own extra- or intracellular-inhibitors. Smad6 and -7 are part of this negative feedback loop and the mouse *Smad6* promoter region has been shown to

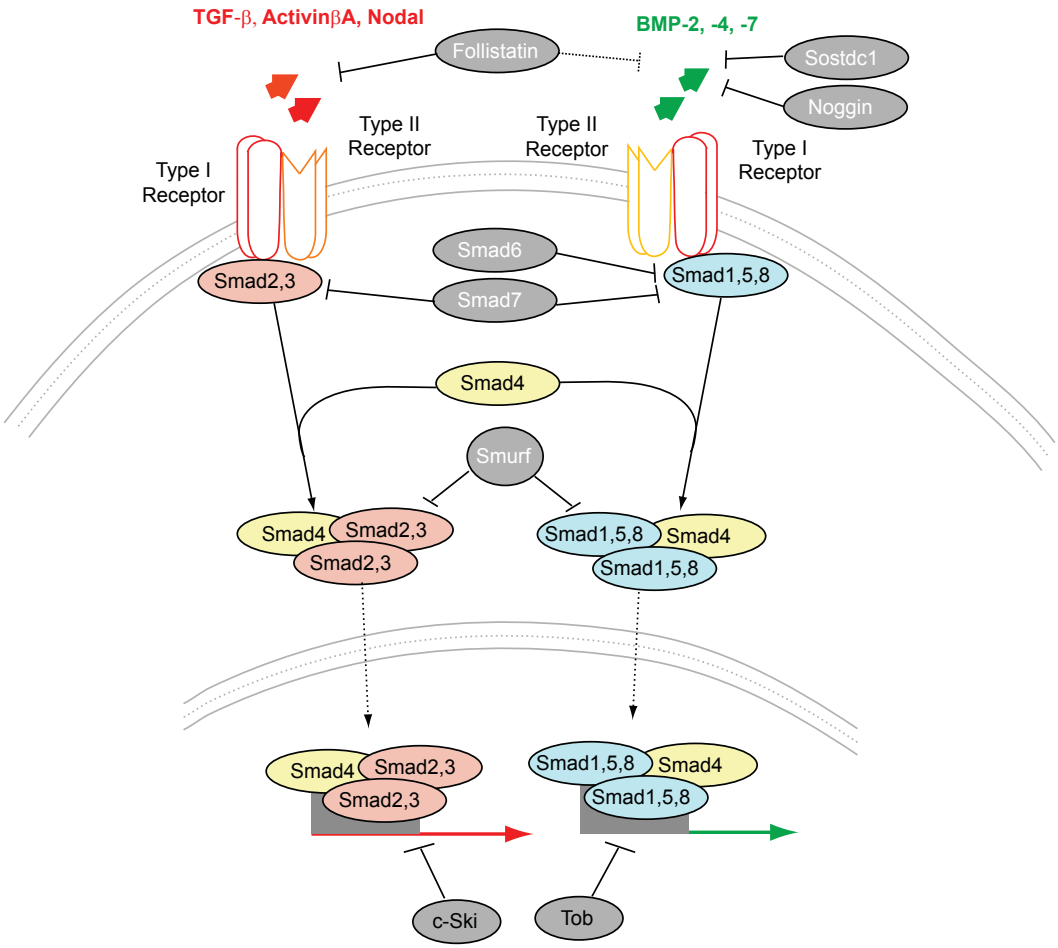


Figure 2. *ActivinβA, Nodal, TGF-β, and BMP ligands bind the cell surface type II receptors. This will phosphorylate the type I receptors and lead to the activation of intracellular R-Smad molecules. ActivinβA activates R-Smad2 and -3 and BMPs activate R-Smad1, -5, and -8. The common Smad4 forms a complex with two R-Smads and translocates into the nucleus to regulate transcription. The signal transduction can be inhibited at many different levels. Extracellularly, there are molecules, such as Noggin and Sostdc1, which can bind to the ligands and prevent their contact with receptors or molecules like Follistatin that disturb the ligand-receptor contact. Intracellularly, there are two different inhibitory Smads that prevent the activation of R- and co-Smads. Smad7 targets all R-Smads, but Smad6 only targets Smad1,5,8. In addition, Smurf1 is able to inhibit the R-Smads intracellularly. Tob and c-ski are nuclear transcriptional inhibitors of the ActivinβA and BMP signaling pathways.*

contain a BMP4 responsive element (**Ishida et al. 2000**). Like morphogens in general, BMPs, are able to activate or repress different genes in a dose-dependent manner (**Gurdon and Bourillot, 2001; Neumann and Cohen, 1997**). The BMP activity depends on its own concentration and the distribution of its antagonists. Low and high dosages often result in opposite cell fate decisions (discussed later in *Craniofacial development*). During tooth development BMP4 regulates the enamel knot activation and induces the differentiation marker, *p21*, in the epithelium (**Jernvall et al. 1998**). In addition, BMP4 induces many target genes such as *Msx1*, *Msx2*, *Lef1*, and *Id1*. The transcription factors, *Msx1* and *Lef1*, are induced in the dental mesenchyme. Transgenic loss-of-function mice have shown their importance for tooth to proceed beyond the bud stage (**Satokata and Maas, 1994, Van Genderen et al. 1994**).

2.6.5 Wnt

Wnt/Wingless is a highly conserved growth factor family that has many different effects such as mitogenic stimulation, cell fate specification, and differentiation. Wnt signaling is mediated through canonical- or non-canonical pathway. Planar cell polarity (PCP), calcium related, and Ror2 or Ryk mediated signaling belong to the non-canonical pathways (**Gordon and Nusse, 2006**). The canonical Wnt pathway is mediated through the stabilization of intracellular beta-catenin. Beta-catenin is also associated with the intracellular domain of E-cadherin and functions as a structural component in cell-cell contact (**Willert and Nusse, 1998**). In the nucleus it interacts with the Lef1/TCF transcription factor to start gene expression as a response to the Wnt signaling (**Behrens et al. 1996**). The absence of *Lef1* blocks tooth development at the bud stage (**Van Genderen et al. 1994; Kratochwil et al 1996**). It is expressed in the thickened dental epithelium at E11 and at later stages both in the mesenchyme and epithelium. Bead experiments have shown that BMP2 and BMP4 are able to induce *Lef1* expression in the dental mesenchyme (**Chen et al. 1996; Kratochwil et al. 1996; Dassule and McMahone, 1998**). A Wnt-responsive element in the *Lef1* promoter allows, as well directs, Wnt regulation (**Filali et al. 2002**).

Many Wnt ligands, receptors, and inhibitors are expressed during tooth development and most of the ligands are expressed only in the epithelium (**Sarkar and Sharpe, 1999; Suomalainen and Thesleff, 2010**). Canonical Wnt signaling appears to be important for the ectodermal organogenesis, since inhibition of Wnt by Dkk1 prevents hair, tooth, and mammary gland formation (**Andl et al. 2002**). Forced activation of the Wnt/β-catenin pathway in the dental epithelium causes supernumerary tooth formation both in the incisor and molar region (**Järvinen et al. 2006; Liu et al. 2008; Wang et al. 2009**).

Wnt signaling begins at the cell membrane after the binding of the Wnt ligand to the Frizzled receptor (Fz). Wnt signaling requires an additional co-receptor, LRP5 or LRP6 (**Pinson et al. 2000; Tamai et al. 2000**). The binding of ligand leads to the inactivation of the zeste-white 3/ glycogen synthase kinase3β (zw3/GSK3β), which normally prevents the accumulation of β-catenin in the cytoplasm. Axin2 is a negative feedback regulator of Wnt pathway. It is activated by β-catenin, which in turn is degraded by the complex of GSK3β and Axin2. The stabilization of the β-catenin is likely to be the key event in the Wnt pathway. The elevation of β-catenin levels leads to its nuclear accumulation (**Cox et al. 1999**). Transgenic reporter constructs containing multimerized TCF binding sites allow the visualization of Wnt signaling (**DasGupta and Fuchs, 1999; Maretto et al.**

2003). These reporter mice, TOPgal and BATgal, are used to visualize the Wnt/β-catenin mediated transcriptional activation. However, these reporter-mice have been shown to fail to label all the sites with known Wnt/β-catenin activity (**Dessimoz et al. 2005; Fathke et al. 2006; Barolo, 2006; Suomalainen and Thesleff, 2010**). The odontogenic mesenchyme is one of these sites with active canonical Wnt signaling that fails to be labeled by reporter gene activity (**Chen et al. 2009**). Chen et al 2009 showed that the mesenchymal Wnt/β-catenin activity is required with BMP2/4 to induce the expression of *Lef1*. Without the mesenchymal Wnt/β-catenin the tooth fails to form, as in the *Lef1*-/- mouse. The signaling is mediated through two families of receptors, Frizzled- and low-density lipoprotein receptor-family (LRP) (**Bhanot et al. 1996; Yang-Snyder et al. 1996; He et al. 1997; Tamai et al. 2000; Pinson et al. 2000**). Wnt antagonists like Dkk1 and Sostdc1 are able to bind the Wnt co-receptor LRP5/6 (**Glinka et al. 1998; Itasaki et al. 2003).** In addition the Sostdc1 binds the negative regulator of Wnt/β-catenin signaling, LRP4 **(Ohazama et al. 2008**). Activation of Wnt signaling has been recently found in many cancers, such as in colon cancer, where Wnt signaling has been shown to target the proto-oncogene c-myc.

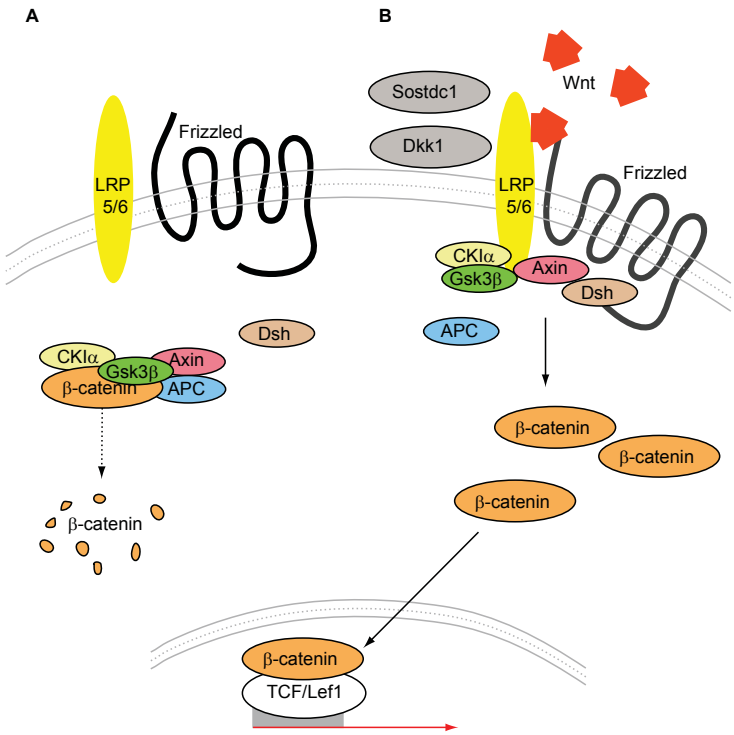


Figure 3. *In the absence of Wnt ligand the intracellular β-catenin is bound by a complex of Axin2, APC, CKIα and Gsk3β, which will target it to degradation (A). The binding of Wnt ligand to Frizzled (Fz) and the Wnt co-receptor Lrp5/6 inhibits the degradation complex by activating the intracellular Dishevelled (Dsh) protein. This will lead to the accumulation β-catenin, which will initiate transcription in the nucleus by interacting with the Lef1/TCF transcription factors (B). Sostdc1 and Dkk1 are extracellular inhibitors of canonical Wnt signaling. They block the activation of the Wnt pathway by binding to the Wnt co-receptor LRP5/6.*

2.6.6. Follistatin

Follistatin is a secreted ActivinβA antagonist (**Nakamura et al. 1990; Hemmati-Brivanlou et al. 1994**), which inhibits BMPs (BMP2, -4, -7) as well, but with a lower affinity (**Iemura et al. 1998; Balemans and Van Hul, 2002**). Microarray analysis and bead experiments have revealed that ActivinβA and Wnt signals induce *Follistatin* expression in the epithelium (**Ferguson et al. 1998, Willert et al. 2002**). Unlike the other extracellular BMP inhibitors, Follistatin does not limit the availability of BMP to its receptor, but forms a trimeric complex with the ligand and receptor causing a conformational change and therefore the inactivation of signaling (**Iemura et al. 1998**). During tooth morphogenesis, Follistatin is an important regulator of BMP and ActivinβA signals. The lingual side of the mouse incisor lacks enamel, which will lead to the uneven wear of the tooth. This is considered to be an adaptation for keeping the cutting edge sharp in the ever-growing mouse incisors. *Follistatin* expression at the lingual incisor epithelium has been proposed to prevent the ameloblast differentiation by antagonizing BMP4 from underlying mesenchymal odontoblasts (**Wang et al. 2004a**). During molar tooth development, Follistatin regulates the morphogenesis of dental epithelium and shaping of the tooth crown (**Wang et al. 2004b**). *Follistatin* is expressed in the primary and secondary enamel knots and in the flanking epithelium. In the absence of *Follistatin*, the molar cusps lack anterior-posterior polarity. In the incisors the enamel develops on both sides of the tooth and the lingual cervical loop is enlarged (**Wang et al. 2004a**). These mice have defects in many organs such as palate, skeleton, whiskers, and muscles and they die before birth (**Matzuk et al. 1995**). In the chick, the introduction of exogenious recombinant protein has revealed Follistatin's role in feather patterning regulation (**Patel et al. 1999; Michon et al. 2008**).

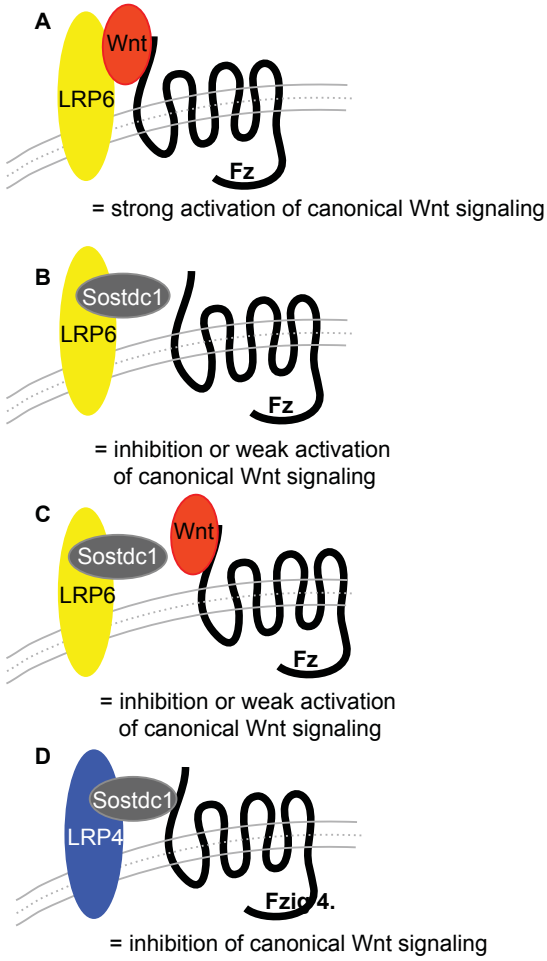
2.6.7. Sostdc1

Sostdc1 is also known as Ectodin, USAG1 and Wise (**Laurikkala et al. 2003; Yanagita et al. 2004; Itasaki et al. 2003**). It belongs to the Dan/Cerberus family of secreted BMP inhibitors (**Laurikkala et al. 2003**; **Balemans and Van Hul, 2002**; **Kusu et al. 2003**; **Avsian-Kretchmer et al. 2004**). It is induced by BMP and it antagonises BMP-2, -4, -6, and -7 with high affinity (**Laurikkala et al. 2003**; **Yanagita et al. 2006**). Sostdc1 is able to modulate the Wnt pathway in a context dependent manner (**Itasaki et al. 2003**). It can activate or antagonize the Wnt pathway by binding the Wnt co-receptor LRP6 and compete with Wnt8 for binding LRP6 (**Itasaki et al. 2003**). It is also able to repress Wnt signaling by blocking the activities of Wnt1, Wnt3a, and Wnt10b (**Yanagita et al. 2004; Beaudoin et al. 2005; Blish et al. 2008**). Interestingly the LRP6-interacting loop domain in Sostdc1 functions independently from the BMP-binding domain (**Lintern et al. 2009**). Sostdc1 can also interact with LRP4, which is considered to be a repressor of Wnt pathway, by competing for LRP5/6 in the Wnt/Frizzled (Wnt/Fz) complex (**Ohazama et al. 2008; Johnson et al. 2005**). In early development, *Sostdc1* is expressed in the first and second branchial arches (**Yanagita et al. 2003**). In addition to the tooth, it is expressed during the development of the vibrissae, hair, feathers, mammary glands, and kidneys (**Laurikkala et al. 2003; Yanagita et al. 2006; Shigetani and Itasaki 2007**). The expression of *Shh* and *Fgf4* in the enamel knot suppresses *Sostdc1* in the signaling center region (**Laurikkala et al. 2003**). During tooth morphogenesis, *Sostdc1* is expressed both in the epithelium and

mesenchyme in a pattern complementary to the expression of *Follistatin* (**Wang et al. 2004b**).

2.7. Crosstalk between Wnt and BMP signaling

Wnts and BMPs are co-expressed in several contexts during development and participate in similar events such as patterning, organ development, stem cell maintenance, and cancer progression (**Itasaki and Hoppler, 2010**). The crosstalk between Wnt and BMP pathway is complicated since the effects vary in a context dependent manner. Both synergistic and antagonistic effects have been reported (**Azpiazu et al. 1996; Carmena et al. 1998**). Mammalian bone formation is an example of such interaction. BMPs together with the canonical Wnt/β-catenin signaling participate specifically in the regulation of bone density through LRP5/6 mediated signaling (**Johnson et al. 2004**). During osteoblast differentiation BMP is required to block Wnt signaling, but after differentiation both pathways can co-operate in the same cells, since the role of Wnt signals in these differentiated cells has changed (**Katagiri et al. 2008**). A loss-of-function mutation in Lrp5 causes osteoporosis whereas overactive *Lrp5* gene leads to high bone mass. BMP2 was shown to stimulate *Lrp5* expression (**Zhang et al. 2009**).



Synergistic pathways can enhance the duration of a signal. The activated Wnt/GSK3β pathway, for example, has been shown to prolong the activity of BMP/Smad1 signaling (**Fuentealba et al. 2007**). Other interactions between the TGF-β and Wnt pathway were reported as a complex formation between the Smad4 and β-catenin (**Nishita et al. 2000**) and Smad4 and Lef1 (**Nawshad and Hay, 2003; Lim and Hoffman 2006; Nawshad et al. 2007**). The TGF-β pathway was shown as well to trigger the nuclear translocation of

Figure 4. *In addition to the ability to inhibit BMPs, Sostdc1 regulates the Wnt pathway in a context dependent manner. Binding of Wnt ligand to Frizzled and the Wnt co-receptor LRP5/6 activates the canonical Wnt pathway (A). Sostdc1 is able to bind the same site on Lrp5/6 as the Wnt ligand leading to the inhibition or weak activation of canonical Wnt pathway (B, C). In addition to LRP5/6, Sostdc1 binds a negative regulator of the canonical Wnt pathway, LRP4, which prevents canonical Wnt signaling (D).*

β-catenin at least in one cell type (**Jian et al. 2006**). If two different signalling networks such as TGF-β and Wnt are connected intracellularly, the availability of a limited pool of Smad4 could affect the efficacy of Wnt signalling or the availability of Smad4 for other TGF-β signal cascades providing different biological responses. Physically separate domains in Sostdc1 protein structure allow the simultaneous antagonistic interaction of BMP and the context dependent modulation of Wnt. Sostdc1 may thus affect the mutual balance between these two important pathways while co-expressed in the same tissue.

2.8. The role of TGF-beta family in the morphoregulation

BMPs are considered as morphogens. They are shown to contribute, depending on concentration, cellular context and timing, in many different kinds of developmental events both in invertebrates and vertebrates (**Wharton et al., 1993; Dale and Wardle, 1999; Dale and Jones, 1999; De Robertis et al., 2000**)**.** BMPs stimulate cell proliferation, apoptosis, and cell differentiation. The development of many structures is shown to be very sensitive to the changes in the BMP concentration, since the low and high levels of BMP4 signaling usually cause opposite cell fate decisions, either proliferation or differentiation (**Plikus et al. 2004**). The repetitive use of the signaling pathways such as BMP at different hierarchical levels, like in feather formation, is suggested to reflect the co-option of the existing molecular pathway to the production of evolutionary novelties (**True and Carroll, 2002; Prum and Dyck, 2003**). This suggests an important role for the BMP4 antagonists in the regulation of pattern formation. The multiple defects in mouse ectodermal organ development after the ectopic Noggin expression under the K14-promoter is suggestive of the multiple roles of TGF-β signaling in the morphological evolution of ectodermal organs (**Plikus et al. 2004**). This inhibition of epithelial BMP increased the number of hairs, reduced size of the claws, and converted sweat glands into foot pads and eye lids into hairs. This partial downregulation of epithelial BMP signaling also affected the development of the dentition (**Plikus et al. 2005**). The tooth number, size, and shape were reduced. The tooth identity did not, however, change, which was contrary to the report of Tucker et al. 1998 showing that exogenous Noggin was able to change the incisor identity into molars.

Mesenchymal BMP4 signaling through epithelial BMPR-IA is required for the activation of signaling centers in the tooth and limb, as well as in limb distal outgrowth (**Ahn et al. 2001; Pizette et al. 2001; Andl et al. 2004; Ovchinnikov, 2006**). Here the studies on limb development were done both in mouse and chicken. This epithelial BMPR-IA activation was shown by a conditional gene deletion to act upstream of epithelial β-catenin stabilization in the apical ectodermal ridge (AER) and in tooth enamel knot formation (**Soshnikova et al., 2003; Andl et al. 2004**). The targeted conditional ablation of the *Bmpr-Ia* receptor in the apical AER severely impaired limb formation, as well as the Wnt responsiveness of the region. Apoptosis has a role in controlling organ shape through the regulation of cell number and the elimination of structures (**Meier et al. 2000**). The function of the signaling centers, like the enamel knot in the tooth and the AER in the limb, will be terminated by apoptosis (**Vaahtokari et al. 1996b; Jernvall et al. 1998**).

During limb development BMPs are reported to participate to pattern formation, interdigital cell death, and bone morphogenesis (**Hogan, 1996**). In the chick limb the *Bmp* expression in the interdigital area seems to correlate with the apoptotic regression of this soft tissue. In the duck this interdigital *Bmp* expression is lacking and leads to the

development of the webbed-feet morphotype. Blocking the *Bmp receptor type IB* (*Bmpr-IB*) in chick by introducing dominant negative *Bmpr-IB* with the viral vector (RCAS) to the hind limb caused a transformation of the phenotype from the chick- to the duck-like limb (**Zou and Niswander, 1996**). In addition, the disruption of *Bmpr-IB* function also caused transformation of the scale in the chick foot into feathers (**Zou and Niswander, 1996**). This change of scales to feathers after blocking the BMP activity represents a transformation of an organ type to another as a response to a change in a morphogen level. Scales and teeth seem to develop in high concentration of BMP, whereas the feather and hair development appears to require low BMP but high Wnt activity. Interestingly, the interdigital overexpression of another member of TGF- family, ActivinA, inhibited the interdigital apoptosis and caused the activation of a chondrogenic cascade leading to ectopic digit formation in chick (**Gañan et al.1996; Merino et al. 1999**). The same was observed by using Noggin beads to antagonize BMPs (**Merino et al. 1998**). This is suggestive of BMPs and ActivinA having opposite roles. Their antagonistic actions may follow from the intracellular competition of a limited pool of Smad4 (**Zimmerman et al. 1998**). Digit development seems to be dependent on ActivinA signaling, except at the tips of the digits. This part of the limb is the only part with regenerative potential (**Borgens, 1982**). In the absence of functional *Bmpr-IB* in chick, the distal development of the digits was affected (**Zou and Niswander, 1996**). Expression analysis and transgenic mouse models have suggested a role for Msx1 and BMP4 as regulators of this distal region (**Reginelli et al. 1995; Han et al. 2003**). Yu et al. 2010 showed that BMPs can induce a regenerative response in mice digits after amputation. Odelberg et al. 2000 showed that Msx1 is required to dedifferentiate the terminally differentiated murine myotube cells. These cells can be de-differentiated into cells expressing chondrogenic, adipogenic, myogenic, and osteogenic markers. In addition, BMP and thus Smad 1/5/8 signaling has been shown to be important in digit identity determination in chick (**Dahn and Fallon, 2000; Drossopoulou et al. 2000; Suzuki et al. 2008**). This was demonstrated with bead experiments and reporter assay. Overexpression of *Bmp2* with a retro viral vector in chicks was reported to duplicate the anterior digits (**Duprez et al. 1996**). In contrast, the conditional deletion of mesenchymal *Bmp4* in mice led to a prolonged exposure of *Fgf8* expression and thus to the posteriorization of the ectopic digits (**Selever et al. 2004**).

2.9. Craniofacial development

Morphogenesis of the mandibular processes seems to be dependent on two independent regions: two large proximal *Fibroblast growth factor8-* (*Fgf8*) and a small medial *Bmp4*-expressing region. Studies from the mandibular processes of conditional *Bmp4*-deficient mice have revealed that the epithelial *Bmp4* expression in the medial region restricts the *Fgf8* expression in the proximal region. It prevents *Fgf8* expression in the distal region and is needed to maintain the expression in the proximal region (**Liu et al. 2005a**). BMP4 was shown to affect pattern formation in dose dependent manner, by inducing different target genes depending on its concentration. The similar dual function of BMP4 as an activator and repressor has been shown in the development of dorsal telencephalon **(Monuki et al. 2001)**. Msx1 was shown as one of the BMP4 targets, whose expression was threshold specific. These studies were based on the differential expression patterns of *Msx1* in genetically

altered mice. Like in limb development (**Wang and Sassoon 1995**), both FGF8 and BMP4 are capable of inducing *Msx1* expression in the mesenchyme (**Vainio et al. 1993**). In mice, the absence of *Msx1* expression disturbs jaw development and causes the complete absence of teeth (**Satokata and Maas, 1994**).

In birds, BMP4 appears to be essential for the mesenchymal proliferation and the outgrowth of facial prominences (**Ashique et al. 2002**). These studies were based on bead experiments, where beads releasing recombinant protein of the BMP antagonist, Noggin, reduced the mesenchymal proliferation and thus the outgrowth of the frontonasal mass. The effect from this temporary mesenchymal BMP block on the overlying epithelium appeared, however, to be opposite and increased epithelial survival. Similarly the introduction of a BMP soaked bead on the chick maxillary primordium increased cell proliferation, which was detected with the BrdU assay, and extended the *Msx1* expressing region (**Barlow and Francis-West, 1997).**

Classic heterospecific recombination studies in birds have revealed that the beak morphology appears to be determined by the cranial neural crest cells (**Schneider and Helms 2003**). *Bmp4* is located in the growth zones of the beak frontonasal mass and the expression in the upper beak neural crest derived mesenchyme correlates with the deep and broad beak morphology in the Galapagos finches (**Abzhanov et al. 2004; Wu et al. 2004**). The deeper beak morphology was also observed in chick after over expressing *Bmp4* with replication-competent avian sarcoma virus (**Wu et al. 2004**). Lee et al. in 2001 showed in their bead implantation study that BMP4 and retinoic acid are needed to specify the identity of the frontonasal mass and maxillary prominences. They introduced BMP antagonist Noggin together with retinoic acid to the developing maxilla, which led to the duplication of the beak due to the transformation of maxillary prominence into a second frontonasal mass. During the later development *Bmp4* expression appears to reside in the forming skeletogenic tissue and especially in their growth centers. Merrill et al. 2008 showed that neural crest-derived mesenchyme and BMP signaling regulate the timing of mandibular osteogenesis and further the evolution of species-specific skeletal morphology in birds. Classic heterospecific transplantation studies uncovered spatiotemporal changes in *Bmp* expression that correlated with the observed differences in the bird beak morphologies. BMP4 has been shown to take part in the regulation of jaw evolution as well in cichlid fishes (**Albertson et al. 2005**). The robustness of the jaw seems to correlate with the expression pattern of *Bmp4* (**Albertson et al. 2003**). The medial *Bmp4* containing region seems to consist of highly proliferative mesenchyme and thus allows the overall growth of the mandible.

In addition to BMP4, Calmodulin1 (CaM1) appears as well to be important during the finch and cichlid fish craniofacial evolution (**Abzhanov et al. 2004; Abzhanov et al. 2006; Albertson et al. 2003; Terai et al. 2002).** Calmodulin1 is a mediator of calcium signaling. It was found to correlate with the long and pointed beak morphology in birds (**Abzhanov et al. 2006**). Unlike *Bmp4*, the overexpression of *Calmodulin1* in the chick only caused the elongation of the upper beak. Parsons and Albertson 2009 showed that *Calmodulin1* is also expressed during jaw development in cichlids and its pattern seems to change according to the jaw morphology. It was shown to be present in the mesenchyme, surrounding the skeletal tissue, suggesting a role in the morphogenesis and formation of the skeleton by modulating the proliferation and differentiation of osteogenic precursor cells (**Zimmerman et al. 1998; von Bubnoff and Cho, 2001; Scherer and Graff, 2000**). Calmodulin1 and

BMP4 can interact through a series of events and their crosstalk has been thought to have an important effect on craniofacial development and evolution.

2.10. Trends in the evolution of dentition

There are different theories for the origin of the dentition. It is believed that before the evolution of jaws, early chordates had dermal tooth-like structures on the outer body wall called odontodes (**Butler, 1995; Smith et al. 1998**). According to the "outside-in-theory", teeth are thought to have evolved from this exoskeleton of early chordates (chondrichthyan fish), when the anterior scales (placoid denticles) were recruited to a new function (**Reif, 1982; Smith and Hall, 1990**). In compliance with some new evidence, the teeth may have also developed more independently from the jaws than previously thought (**Smith, 2003**). This "inside-out" theory is based on the pharyngeal teeth present in many jawless fish that resemble the jaw teeth and are therefore considered to precede them in phylogeny. Recently Huysseune et al. 2009 revised this "outside-in"-theory and suggested that teeth may have arisen before the formation of the jaws, when the odontode-forming ectoderm invaded to the oropharyngeal region and interacted with the neural crest derived mesenchyme. This may give further support to the homology and developmental similarity between the skin denticles and teeth. The molecular similarity between the teeth forming from the pharynx and stomodeum suggests that the molecular machinery needed for the tooth production evolved only once, although their epithelial origin is different (oral ectoderm in the oral teeth and endodermal epithelium in the pharyngeal teeth). The neural crest derived mesenchyme and its odontogenic potential is therefore considered as a key innovation in the evolution of teeth (**Soukup et al. 2008**).

After the development of the jaws, the dentition was thought to be homodont for quite a long time. However, the morphological complexity of teeth from homodonty to heterodonty was thought to start to increase at the same time (**Peyer, 1968; Luckett, 1993**). In some vertebrates like teleost fishes, tooth replacement occurs, however, without the continuous dental lamina and each successional tooth forms from the outer dental epithelium of the older tooth germ (**Fraser et al. 2004**). The evolutionary pathway from fish to reptiles to mammals seems to be characterized by a reduction in the number of teeth. The morphological complexity of teeth from homodonty to heterodonty was thought to start, however, to increase at the same time (**Peyer, 1968**). Other segmented systems like limbs are thought to have undergone a similar transition, where the regional information has changed the morphology of the segmental structure to be more diverse (**Weiss, 1990, 1993**). The development of heterodonty is more characteristic for the evolution of mammals, although some fishes and mammalian-like reptiles (proto-mammals) already had some multicusped teeth (**Peyer, 1968; Osborn, 1973**). The evolution of the multicusped tooth has been debated since 19th century. For the moment the first cusp appearing during ontogeny, the protoconid in the lower molars, is considered to be the original cusp in the evolution of multicusped teeth. The initiation of other cusps may vary between teeth and species (**Butler, 1956**). According to the concrescence theory, multicusped teeth may have evolved through the fusion of several simple cusped teeth during the reptile-mammalian transition (**Kükenthal, 1892; Röse, 1892; Adloff, 1916**). This theory, however, is generally not regarded as valid

in the evolution of synapsid dentition, where the transition from simple teeth to complex multicusped teeth is well documented (e.g., **Carroll, 1988**).

During the evolution of placental mammals, the loss of teeth is thought to be the most common change in the dentition (**Peyer, 1968**). The ancestral dental formula for placental mammals is considered to be three incisors, one canine, four premolars, and three molars in half a jaw (**Carroll, 1988**; **Luckett, 1993**) and the epithelial extension of the primary tooth epithelium was thought to be the source for the secondary tooth formation. Interestingly, the evolutionary decrease in tooth number has been considered to occur in reverse order to their appearance in development (**Weiss et al. 1998**). The loss of the structures from the tetrapod limbs, seem to follow a similar pattern (**Hinchliffe 1994; Shubin et al. 1997**).

2.11. Mouse dentition

The mammalian dentition consists of four tooth families: molar, premolars, canines, and incisors (**Butler, 1956; Osborn, 1978**). The number of teeth and the tooth shape varies between species (**Evans et al. 2007**). Incisors in insectivores can be enlarged like in hedgehogs or cusped like in shrews, presumably to enable better capture of the prey. One of the main functions of vertebrate teeth is to obtain and process food. They may also take part in other actions like defense for instance. The size of teeth is also variable among species and some teeth can reach enormous sizes like elephant tusks. The large continuously growing incisor is a distinctive feature of mice and other gnawers. The epithelial stem cell niche is suspected to be in the cervical loop at the basal end of the incisor and it is thought to allow the continuous growth of this rootless hypsodont (hypselodont) tooth (**Harada et al. 1999**). The labial cervical loop appears to be enlarged and is thus considered to maintain a faster growth rate compared to the lingual side. These stem cells give rise to the enamel forming ameloblasts (**Harada et al. 1999**). The enamel is only deposited on the labial surface of this single cusped incisor tooth, which is thought to enable the uneven wear of the tooth and therefore keeps the cutting edge sharp (**Wang et al. 2004a**). This could be considered as a key adaptation of rodents since it allows the consumption of wide variety of food. A similar dentition is found in the Order Lagomorpha. Evolutionary relationship between Lagomorpha and Rodents has been proposed (**Van Valen, 1966; Meng et al., 1994**) and they are typically considered to belong to the same clade, Glires, in recent literature (e.g., **Asher et al. 2005**).

In the mouse, the number of teeth is reduced containing only three molars and one incisor in every jaw quadrant. The canines and premolars have been lost through the evolution leaving a toothless gap, called a diastema, between molars and incisors. Rudimentary tooth germs are, however, observed in the mouse diastema region during early embryonic development. These rudiments have been shown to develop to the early bud stage, but to disappear later through apoptosis (**Tureckova et al. 1995; Keränen et al. 1999**). The transient expression of some signaling molecules (*Shh*, *Lef1*, *Msx1*, *Msx2*, *Bmp2*, and *Bmp4*) in the mouse diastema epithelium suggests the retention of tooth forming potential (**Keränen et al. 1999; Tureckova et al. 1995**). Mesenchymal *Bmp4* and other mesenchymal genes are downregulated in the diastema buds (**Keränen et al. 1999**). Yuan et al. 2008 showed that the loss of inductive potential from the diastema mesenchyme at E13 may have caused the suppression of the diastema rudiments during development. This

may have followed the appearance of inhibitory molecules. Additionally, the deciduous dentition is normally replaced by the permanent teeth in most mammals, but mice lack tooth replacement.

During the early development of the rodent dentition the *Fgf8* expressing proximal domain (laterally) and *Bmp4* expressing distal domain (medially) are considered to divide the mouse lower jaw into two domains. According to bead experiments, BMP4 is known to induce the mesenchymal expression of *Msx1* and *Msx2*, whereas FGF8 induces *Msx1*, *Pax9*, *Barx1* and *Dlx2* (**Neubuser et al. 1997; Tucker et al. 1998; Trumpp et al. 1999; Ferguson et al. 1998**). The tooth position and identity are considered to follow these differential expression profiles between the proximal and distal domains. BMP4 induces *Msx1* expression in the mesenchyme, whereas FGF8 is able to induce both *Msx1* and *Pax9*. This enables the mesenchymal *Bmp4* expression needed for enamel knot activation (**Andl et al. 2004; Liu et al. 2005a; Peters and Balling, 1999**). The absence of *Msx1* or *Pax9* causes tooth agenesis, which reflects their key role in the development. The inhibition of BMP4 by Noggin in the distal mesenchyme at E10 was shown to cause an ectopic expression of *Barx1* in the incisor region and thus it was suggested to change the tooth identity from incisors into molars (**Tucker et al. 1998**)

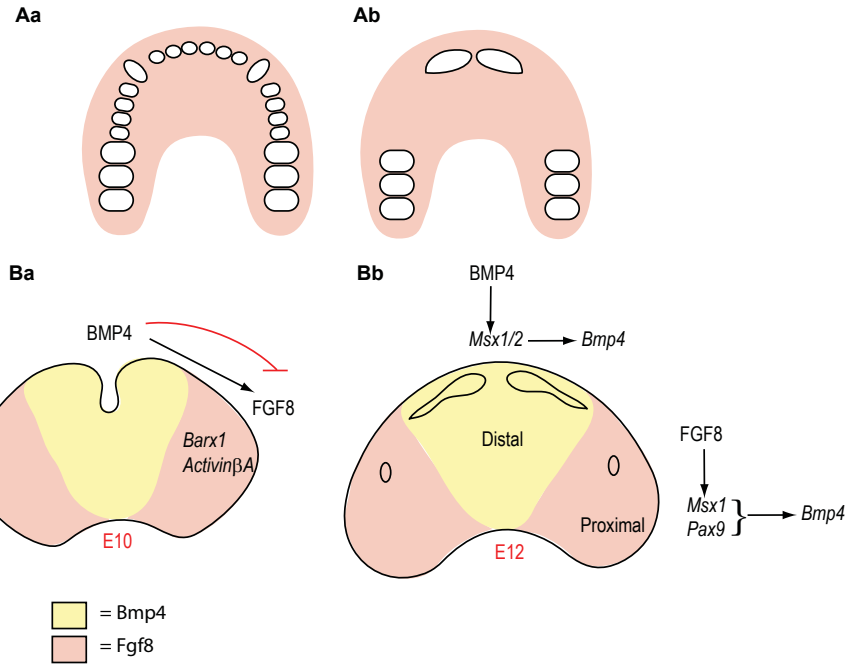


Figure 5. *The general eutherian dental formula consists of three incisors, one canine, four premolars, and three molars in a jaw quadrant (Aa). In rodents, there are only one large incisor in the distal and three molars in the proximal area (Ab). During jaw development, the expression of Bmp4 and Fgf8 divides the jaw in two regions: the distal incisor region and the proximal molar region (Ba). BMP4 affects the expression of Fgf8 in a dose-dependent manner: a high concentration inhibits Fgf8, but a low concentration is required for Fgf8 expression. Both FGF8 and BMP4 are able to induce Msx1 expression in the mesenchyme among several other genes. FGF8, for example, is required for the mesenchymal Barx1 and ActivinβA expression.*

2.12. Supernumerary teeth in rodents

Tooth loss seems to be far more common than the appearance of supernumerary teeth. Grüneberg, in 1951, suggested that there is a critical size for the tooth primordium. If the primordium is too small, the tooth fails to develop. Vestigial tooth structures are thus eliminated, suppressed or incorporated into the functional dentition. The rudimentary teeth in mutant mice may be atavistic structures that appear when the developmental program that is normally suppressed becomes reactivated (**Peterkova et al. 2006**). Many genetically altered mice strains show supernumerary tooth formation. Predominantly, this extra tooth forms anterior to the first molar at a position where there is a known rudimentary tooth bud (**Mustonen et al., 2003; Zhang et al. 2003; Kangas et al. 2004; Klein et al., 2006**; **Ohazama et al. 2009**). This extra tooth seems typically to have the appearance of a premolar. According to some studies, the disappearance of the last premolar correlates with the mesial enlargement of the first molar in mouse (**Peterkova et al. 2005**). This extra tooth may thus represent a rudimentary premolar that is incorporated at the mesial part of this first molar in the modern mouse (**Peterkova et al. 2002**).

Supernumerary incisors in rodents appear to be rarer, but have been reported at least from rats, lemmings, squirrels, and mice (**Hansen, 1956**). In squirrels (rodent family Sciuridae), two small maxillary and mandibular vestigial permanent incisors are reported to form, but they seem to fail to develop beyond the early bell stage (**Luckett, 1985**). The upper incisor in rats appears to form from two primary dental placodes located in different facial processes. If the fusion of these processes is incomplete like in the *Pax6*-deficient rats, the two placodes remain separate and form two separate incisors (**Kriangkrai et al. 2006)**. In mice, supernumerary incisors appear to form in two different positions. They are either located postero-laterally to the main incisor or at the lingual side, similar to rabbits (**Danforth, 1958**; **Peters and Strassbourg 1969; Sofaer, 1969; Moss-Salentijn, 1978).** The mouse gnawing incisors are considered to correlate with the middle incisors of the general eutherian dental formula (I_3, C, P_4, M_3) (**Hershkovitz, 1976; Strassbourg, 1970**). The two others (I_1, I_3) are thought to have disappeared during the evolution of rodents (**Hershkovitz 1976**; **Meng et al. 2003**; **Asher et al., 2005**). During the development of the mouse upper incisors, three different epithelial thickenings are observed (**Strassburg et al. 1971**). Later Peterkova et al. 1993 observed that the whole large incisor epithelial area invaginates into the mesenchyme and seems to give rise at least to 5-6 placodes in the maxilla and around four placodes in the mandible (**Peterkova et al. 1993**). They suggested that the mouse upper incisor may form through a fusion of several primary dental primordia and therefore represents a composite structure. The observed placodes may represent the ancestral incisor formula with five upper and four lower incisors (**Ji et al. 2002**).

Rodents are considered to have lost the ability for tooth replacement. The deciduous dentition is characterized by an earlier development and a replacement by the permanent teeth, which form from their lingual side (**Berkowitz, 1968**). According to the predominant rule of tooth loss in evolution, the permanent teeth are suppressed first as the deciduous teeth are not replaced but rather retained as a part of the functional dentition (**Ziegler, 1971**). All vestigial teeth in mice, rats, and rabbits are considered as deciduous (**Moss-Salentijn, 1975; Moss-Salentijn 1978**), although their relationship to the functional dentition still remains under debate. The observation of minute tooth on the labial side of the main incisor (notably from the mouse maxilla) led to the conclusion that the gnawing incisors represent

the permanent teeth, whereas the deciduous incisors represent embryonic rudiments (**Woodward, 1894; Hinrichsen, 1959**; **Fizgerald, 1973; Peterkova et al. 2006**). Some studies claimed, however, that the mouse fails to develop permanent incisors (**Woodward, 1894; Fitzgerald, 1973; Peterkova et al. 2002**).

The molecular mechanisms leading to extra teeth seem to be diverse. During rodent tooth development, some genes such as *Bmp4*, *Lef1*, and *Msx2* appear to be expressed at E13-14 on the buccal bias of the tooth (**Keränen et al. 1998**). A mouse mutant lacking the transcription factor *odd-skipped-related-2* (*Osr2*) has supernumerary teeth forming from the lingual side of the molars (**Zhang et al. 2009**). *Osr2* is normally expressed on the lingual side of molar mesenchyme, where it was suggested to repress mesenchymal *Bmp4* expression. Mesenchymal BMP4 seems to have an important role in the activation of sequential tooth formation by expanding the odontogenic field on the lingual side. This led to the expansion of *Bmp4, Lef1*, *Msx1*, and *Pitx2* on the lingual aspect. *Bmp4* and *Pitx2* appear to be associated with the tooth replacement mechanism in fishes (**Fraser et al. 2006**). Järvinen et al. 2009 have shown that replacement teeth in ferret form from the dental lamina that is intimately connected to the lingual side of the deciduous tooth. Therefore it is not clear whether these supernumerary molars in *Osr2*-deficient mice represent the replacement teeth or the expansion of epithelial odontogenic potential and perhaps a second row of teeth (**Mikkola, 2009**). One of the most striking mutants with supernumerary tooth formation is seen after the forced epithelial Wnt/beta-catenin signaling (**Järvinen et al. 2006; Liu et al. 2008; Wang et al. 2009**). The first signs of abnormal tooth morphogenesis appear at E13 when ectopic enamel knots have started to form both in the incisor and molar regions. It was impossible to conclude whether these extra teeth were formed successionally or by a subdivision of the dental epithelium. In humans the mutation in the Wnt pathway antagonist APC is reported to lead to the formation of odontomas, odontogenic "tumors".

Table 2. *Mouse mutants exhibiting supernumerary teeth.*

Mutants	Pathway	Tissue	Phenotype	References
$Pax6^{Sey}$ -/-	unknown	epithelium	Supernumerary incisors in the maxilla.	Kaufman et al. 1995.
$Tg737^{orpk}$ hypomorph	Shh	mesenchyme	Premolar mesial to first molar.	Zhang et al. 2003; Ohazama et al. 2009.
Wnt1-Cre; $Polaris^{flox/flox}$	Shh	mesenchyme	Premolar mesial to first molar.	Ohazama et al. 2009.
Spry2−/−	FGF	epithelium	Premolar mesial to first molar.	Klein et al. 2006; Peterková et al. 2009.
Spry4−/−	FGF	mesenchyme	Premolar mesial to first molar.	Klein et al. 2006.
Tabby+/−	Eda	epithelium	Premolar mesial to first molar.	Grüneberg, 1966; Sofaer, 1969.
K14-Eda	Eda	epithelium	Premolar mesial to first molar.	Mustonen et al. 2003; Kangas et al. 2004.
K14-Edar	Eda	epithelium	Premolar mesial to first molar.	Pispa et al. 2004; Tucker et al. 2004b.
Osr2−/−	BMP	mesenchyme	Lingual molars.	Zhang et al. 2009
Sostdc1−/−	BMP/Wnt	mesenchyme/ epithelium	Supernumerary incisors and premolar mesial to first molar.	Kassai et al. 2005; Yanagita et al. 2006; Murashima-Suginami et al. 2007, 2008; Munne et al. 2009.
Lrp4−/− (Megf7) hypomorph	Wnt	epithelium	Supernumerary incisors and premolar mesial to first molar.	Ohazama et al. 2008.
Epiprofin−/−	Wnt	epithelium	Multiple incisors and molars.	Nakamura et al. 2008.
K14-Cre; $Apc^{cko/cko}$	Wnt	epithelium	Multiple incisor and molar tooth buds.	Kuraguchi et al. 2006.
K14-Cre^{8Brn}; $Apc^{cko/cko}$	Wnt	epithelium	Numerous labial and lingual incisors and molars.	Wang et al. 2009
K14-Cre^{1Amc}; $Apc^{cko/cko}$	Wnt	epithelium	Numerous epithelial tooth buds.	Wang et al. 2009
K14-$CreER^{TM}$; $Apc^{cko/cko}$	Wnt	epithelium	Numerous labial and lingual incisors.	Wang et al. 2009
K14-$CreER^{TM}$; $Ctnnb1^{(ex3)/fl+}$	Wnt	epithelium	Numerous labial and lingual incisors and molars.	Wang et al. 2009
K14-Cre/+; β-$catenin^{Aex3fl/+}$	Wnt	epithelium	Multiple incisor and molar epithelial invaginations.	Järvinen et al. 2006.
K14-Cre; $Ctnnb1^{(ex3)fl/+}$	Wnt	epithelium	Multiple molar epithelial invaginations.	Liu et al. 2008
K14-Lef1	Wnt	epithelium	Ectopic tooth like structures.	Zhou et al. 1995.

2.13. Dollo's law and the timing of development

> *"An organism is unable to return, even partially, to a previous stage already realized in the ranks of its ancestors." – Louis Dollo*

Darwin, in 1859, suggested that the disuse of an organ through generations is a requirement for the gradual loss of structures. In the fossil record, the early step in the process of the evolutionary loss of structures includes the reduction in size and simplification in shape (**Ziegler, 1971**). The degeneration of a structure leads to formation of a rudiment. During development, the ancestral structures are kept, modified or suppressed. The structure may still be present during the early development as a vestigial structure and disappear later through apoptosis (**Moss-Salentijn, 1978**). Dollo's law states that during evolution the lost structures are not able to return exactly as they were. This is based on the assumption that the genetic and developmental mechanisms are too complex to evolve back in a similar manner if lost. According to this law, the genes or developmental pathways, released from the selective pressure, will rapidly become nonfunctional. One implication of the law is that non-functional genes may accumulate mutations or the gene expression may decrease to a lower level than the threshold level needed for development (**Marshall et al. 1994**). There are, however, some examples defying the law. In lynx, for example, the third cusp at the hind edge of the carnassial teeth (M1) as well as second molar (M2) are considered as structures that have re-evolved (**Kurtén, 1963**). Kurtén suspected that this reversal may have followed from changes in the level of gene activity controlling the size of tooth forming region, instead of the re-activation of silenced genes. Although Dollo's Law states that the evolutionary reappearance of lost structures is unlikely, recently lost structures could still re-appear (**Marshall et al. 1994**). This was based on an estimation of the time period that the coding sequence of a silenced gene stays functional and the gene function can be reactivated.

Heterochrony is an evolutionary change resulting from a change in the timing of a developmental event or process (**Richardson, 1999**). Gould in 1977 postulated heterochrony as a mechanism to create macroevolutionary novelties through rapid changes in morphology. Different phenotypes may result, for example, from a different exposure time to growth factors. Heterochrony has been shown to cause re-appearance of ancestral structures. This has been seen, for instance, in some gastropod species, where the change in developmental timing has allowed them to gain back their coiled shell from un-coiled state (**Collin and Cipriani, 2003**). There seem to be many ways of losing structures during evolution, which affect the probability of how easily these structures are able to re-appear.

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3. AIMS OF THE STUDY

During evolutionary history the dentition has undergone different modifications. The correct size, shape, and the number of the teeth are crucial for the dentition to function optimally. How do these changes take place during the mouse embryonic development and how are they regulated at the molecular level? We studied *Sostdc1* gene during the development of mouse dentition as it influences tooth number, size, and morphology. The specific aims of the study were:

1) To investigate the role of Sostdc1 in the development of mouse dentition.
2) To determine the role of Sostdc1 in the regulation of mouse molar shape.
3) To investigate the origin of the supernumerary incisors in the *Sostdc1*-deficient mice and the molecular mechanism behind their development.
4) To examine the underlying developmental mechanisms in the large rodent incisor formation.

4. MATERIALS AND METHODS

4.1 Mouse strains

Mouse strain	Used in article	Purpose
NMRI	I, II, III	mRNA expression studies, *in vitro* cultures, apoptosis studies.
Shh-GFPcre (NMRI)	II, III	mRNA expression studies, *in vitro* cultures.
Sostdc1 -/- (C57BL/6)	I, II, III	Analysis of *Sostdc1*-deficient phenotype.
Follistatin -/- (C57BL/6)	III	Analysis of *Follistatin*-deficient phenotype.
Nude	III	Kidney transplantation experiments.
Tabby	III (unpublished)	*In vitro* cultures.
Caten$\beta^{\Delta ex3k14/+}$	II	Activation of β-catenin in the dental epithelium. *In vitro* cultures.
BATgal	II	Wnt reporter analysis.
TOP-gal	II	Wnt reporter analysis.

4.2 Probes

Probe	Used in article	Reference
Bmp4	II	Vainio et al. 1993
Follistatin	III	Wang et al. 2004b
p21	I, II	Jernvall et al. 1998
Shh	I, II, III	Vaahtokari et al. 1996
Sostdc1	I, II, III	Laurikkala et al. 2003
Barx1	III	Mitsiadis et al. 1998
Msx1	I, III	Vainio et al. 1993
Msx2	II	Jowett et al. 1993
Fgf4	I	Jernvall et al. 1994
Edar	I	Laurikkala et al. 2001

4.3 Methodes used in articles

Methode	Used in article
Tissue culture	I, II, III
Tissue culture with protein (bead)	I, II, III
Tissue culture with protein (media)	I, II, III
Microinjection	II
Histology	I, II, III
Radioactive in situ hybridization on sections	I, II, III
Whole mount in situ hybridization	I, II, III
LacZ staining	II
Tunel-staining	II
Analysis of adult mouse tooth phenotype	I, II
3D analysis of tooth development	I, II
NIH-Image software to measure the expression domain areas	I

5. RESULTS AND DISCUSSION

5.1. The role of Sostdc1 in the molar cusp delineation (I)

We investigated the role of Sostdc1 in the regulation of tooth shape. Previously *Sostdc1* had been shown to be a feedback inhibitor of BMP signaling and a context dependent Wnt modulator (**Laurikkala et al. 2003; Itasaki et al. 2003; Yanagita et al. 2004**). Laurikkala et al. 2003 showed that *Sostdc1* was expressed during tooth morphogenesis around the signaling centers, enamel knots. The expression was, however, several epithelial and mesenchymal cell layers away from the enamel knot, which was suggested to be due to the antagonistic action of Shh and Fgfs expressed in the enamel knot. This peculiar expression pattern and the dynamics between the enamel knot genes made Sostdc1 an interesting molecule, considering the mechanisms regulating enamel knot function and tooth shape.

The *Sostdc1* expression was observed around the enamel knots, when we analyzed the pattern during the mouse molar morphogenesis at E16. We analyzed the tooth phenotype of *Sostdc1* null mutant mice generated by N. Itoh (Kyoto). These mice were fertile and only the null mutant mice represented changed phenotype with interesting dental modifications including altered molar cusps and extra teeth. The complete deletion of the *Sostdc1* during the tooth morphogenesis altered the cusp pattern and led to the decreased separation of cusps and teeth. The morphological phenotype was in line with aberrant function of the enamel knots previously shown in the laboratory to regulate cusp patterning. The *Sostdc1*-deficiency had variable tooth phenotypes. An extra molar was observed to form anteriorly to the first molar. The cusp pattern in this extra tooth varied from very simple to close to the pattern observed normally from the first molar. Around 40% (21/52) of the analyzed skulls had a fusion between first and second molar and some samples had a fusion between cusps at their buccal side. Although the extra cusps were observed to form at the lingual side of the first molar, the buccal side seemed to be the most affected. This may follow from the buccal biased *Bmp4* expression in the molar region and suggest an important role for Sostdc1 in the lateral development of the molar. High-resolution three-dimensional laser-confocal was used to image the differences in crown morphology.

We used *p21*, the earliest differentiation marker expressed in the enamel knot, to indicate the sites of the forming cusps in the mutant embryos. 3D-reconstruction of the radioactive *in situ* hybridization showed the expanded expression pattern of *p21* in the *Sostdc1*-deficient molars. The opposite expression patterns of *Sostdc1* and *p21* in the epithelium suggested a role for Sostdc1 in the delineation of enamel knot activity. As the *p21* expression has earlier been shown to be a BMP4 target (**Jernvall et al. 1998**), we tested if *Sostdc1* could antagonize *p21* induction in the epithelium. We placed an affi-agarose BMP4 soaked bead on isolated molar epithelium of E13 wild type mouse *in vitro*. This led to the up-regulation of BMP4 downstream targets, *p21* and *Sostdc1*. When BMP4 beads were placed on the tooth epithelium together with the Sostdc1 soaked beads the *p21* expressing area decreased. In order to confirm the association of BMP4 to the altered cusp pattern in *Sostdc1*-deficient mice, we tested the effects of recombinant BMP4 in the tooth cultures. As the mesenchymal BMP4 is required for the enamel knot activation, we cultured isolated E13 - E14 *Sostdc1* null-mutant and wild type molars with recombinant BMP4 protein. The enamel knot area was enlarged as a response to the BMP4 treatment in *Sostdc1*-deficient molars and the crown development was accelerated by three days. No difference in the *in vivo* development rate between the wild type and *Sostdc1* null

mouse was, however, observed probably due to the same endogenous BMP4 level. As the mesenchymal BMP4 was able to interact with the overlaying epithelium without limitation in the *Sostdc1*-deficient mouse, the larger epithelial area was suspected to be prone to the enamel knot induction and thus to the expansion in the enamel knot size. As the tooth morphology in the *Sostdc1*-deficient mice was severely altered this suggested an interesting role for *Sostdc1* in cusp area delineation.

We analyzed the origin of the extra molar by investigating the initiation of tooth development using *Shh* as a marker of the dental primordium. Ectopic *Shh* expression at the anterior side of the first molar was observed at the E13.5 in *Sostdc1*-deficient as well as in wild type mice. Kangas et al. 2004 showed this ectopic expression in the wild type indicated rudimental tooth, which normally disappears, but is rescued in some genetically altered mice such as *Sprouty*-deficient or *Ectodysplasin*-overexpressing mice. This extra molar is considered to represent a vestigial premolar that was lost 45-100 million years ago. Despite this evolutionary disappearance, mouse embryos seem to have retained potential to form this tooth (**Meng et al. 1994; Ji et al. 2002**). In *Sostdc1*-deficient mice this rudimental tooth primordium expressed strongly *Shh*. We showed that the absence of *Sostdc1* during morphogenesis led to the expansion of the enamel knot region and enlarged expression patterns of genes in the area, e.g. the Ectodysplasin receptor *Edar*. This may partly explain why there are some phenotypic similarities between *Ectodysplasin* over-expressing mice and *Sostdc1*-deficient mice, such as longitudinal crests connecting lingual and buccal cusps and extra tooth formation.

In many different developmental steps Wnt and BMP pathways seem to work up- and downstream of each other. Mesenchymal BMP4 signaling is required for the enamel knot activation and for the nuclear localization of β-catenin in the epithelium through BMPR1A activity in the enamel knot region (**Andl et al. 2004**). Activated epithelial Wnt signaling will further lead to the *Shh* and *Bmp4* expression in the enamel knot (**Järvinen et al. 2006; Närhi et al. 2008**). In the *Sostdc1*-deficient mice the epithelial localization of nuclear β-catenin was observed to increase together with BMP signaling (**Murashima-Suginami et al. 2008**). *Sostdc1* will be delineated outside the signaling center by FGF and Shh from the enamel knot. This expression pattern around the enamel knot allows Sostdc1 to limit the signals coming from the signaling center such as canonical Wnt pathway and BMP4. Ohazama et al. 2008 showed that loss of the negative co-receptor of Wnt pathway, *Lrp4*, from the enamel knot caused the similar phenotype with the *Sostdc1*-deficient mice. They proposed that Sostdc1 binds BMP4 and simultaneously prevents the activation of canonical Wnt signaling by

Figure 6. *Sostdc1-deficient molars from E13 and E14 stage treated with recombinant BMP4 protein. The introduction of recombinant protein in the culture medium enlarged the enamel knot area, seen here as an expanded Shh expression. The second enamel knot at the E14 stage samples represents the rudimental extra molar. Scale bar 1mm.*

binding to the LRP4 receptor. Interestingly the expression patterns of *Lrp4* and *Sostdc1* are not overlapping, but complementary. This negative regulator of canonical Wnt signaling, *Lrp4*, is expressed in the enamel knot and may thus allow in the null-mouse increased Wnt signaling and thus expansion of enamel knot region. In the *Sostdc1*-deficient mice, the enamel knot area is as well enlarged, but unlike the *Lrp4*- expression, the *Sostdc1* is in the mesenchyme and around this signaling center in the epithelium. The unrestricted mesenchymal BMP4 signaling in Sostdc1-deficient mouse may lead to the enlarged enamel knot induction and together with the missing epithelial Sostdc1-expression to expanded enamel knot region with increased Wnt signaling. Whether the interaction between the LRP4, BMP4, and Sostdc1 is straight as Ohazama et al. 2008 proposed or the similarity between *Lrp4*- and *Sostdc1*-deficient mice is caused by their different, but parallel action during the tooth morphogenesis.

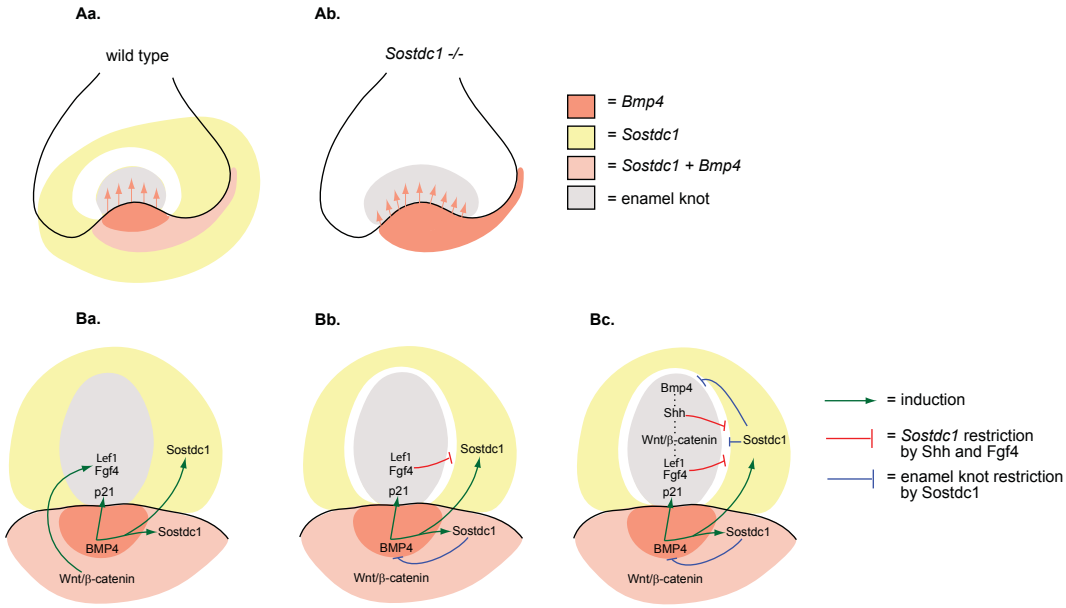

Figure 7. *Sostdc1 restricts the enamel knot size. Mesenchymal BMP4 is required for the enamel knot activation and for the induction of its negative feedback regulator Sostdc1 around the enamel knot (Aa). In the absence of Sostdc1 the enamel knot area enlarges (Ab). The schematic drawing illustrates the delineation of enamel knot by Sostdc1 (Ba,b,c). Mesenchymal BMP4 induces Sostdc1 expression both in mesenchyme and epithelium. It induces as well the first differentiation marker, p21, in the enamel knot (Ba). The epithelial FGF4 prevents Sostdc1 expression in the enamel knot region (Laurikkala et al 2003). Sostdc1 limits p21 expression by restricting the mesenchymal BMP4 signaling (Bb). The enamel knot activity will lead to the Wnt/β-catenin signaling in the enamel knot and to Shh and Bmp4 expression. Sostdc1 may limit the enamel knot activity as well by antagonizing epithelial Bmp4 and Wnt signaling (Bc).*

5.2. Suppression of vestigial incisors by Sostdc1 (II)

The extra tooth forming in front of the first molar in genetically altered mice, e.g. K14-EDA, Tg737^{orpk}, Sprouty- and Gas1-null mutant is suggested to be a rudimental premolar (**Mustonen et al. 2003; Zhang et al. 2003; Klein, 2006; Ohazama et al. 2009**). These mice, however, have no regularly forming supernumerary incisors. In *Sostdc1*-deficient mice, we observed, in addition to the extra molar, supernumerary incisors forming in both the upper and lower jaws. This made *Sostdc1* as an interesting gene for the incisor region development.

We investigated the origin of these extra incisors at the E14 stage and found a strong apoptotic center at the lingual epithelium in the wild type main incisor, but not in the *Sostdc1*-deficient mice. Murashima-Suginami et al. 2007 also reported the supernumerary incisor phenotype from *Sostdc1*-deficient mice, but unlike them, we did not observe changes in mesenchymal apoptosis. Instead we found a correlation between sites of the epithelial apoptosis with an ectopic signaling center activity. TOP-gal and BAT-gal Wnt-reporter mice showed enamel knot activity at the lingual 8pithelium of both wild type as well as *Sostdc1*-deficient incisors at E13.5 stage. This was concluded to indicate an existing potential to form a rudimental tooth, the formation of which is prevented during the development of modern mouse dentition. We used the 3D analysis of serial sections to confirm the origin of these extra incisors. Our study indicated that the small ectopic domain of Wnt-reporter activity at the lingual main incisor epithelium correlated with the site of intense apoptosis.

We investigated the role of *Sostdc1* in the incisor region and found that it was expressed in the mesenchyme at the E12 and E13 stages. Epithelial expression was upregulated mostly at the E14 stage. At the E13 *Sostdc1* expression was only found in the mesenchyme where

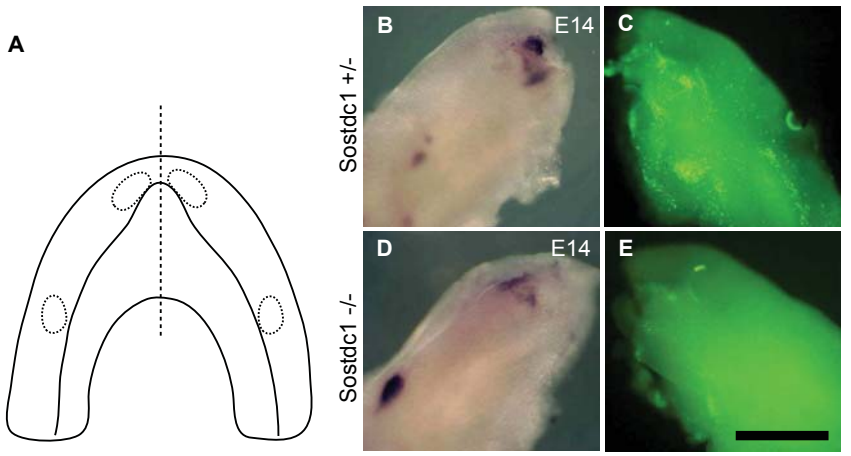

Figure 8. *The schematic drawing illustrates the separation site in the mouse lower jaw at E14 stage (A). TUNEL-staining reveals a strong apoptotic center at the wild type mouse incisor region at E14 stage (C). This is not observed from the Sostdc1-deficient mice, which seem to lack almost the whole epithelial apoptosis (E). The apoptosis at the molar region correlates with the observed premolar rudiment, which is visible as an ectopic Shh expression site in the wild type jaw (B). In the Sostdc1-deficient mice the Shh expression appeared to be stronger compared to the wild type. Scale bar 0.5mm.*

the rudimental incisors will disappear from the epithelium at E14 stage. This suggested that mesenchymal Sostdc1 could inhibit a key factor needed for enamel knot activation. This was supported by the notion that in the *Sostdc1*-deficient mice the enamel knot area was expanded. As the mesenchymal Sostdc1 seemed to restrict the size of forming enamel knots, we wanted to see if reduction in the incisor-surrounding mesenchyme will rescue the rudimental incisor formation in wild type samples. This led to the reduction of *Sostdc1* expression around wild type tooth germs and to the formation of extra incisors *in vitro*. The trimming allowed phenocopying of the *Sostdc1*-deficient phenotype when the incisors were dissected from E12 or E13 stage embryos. The reduction in the surrounding mesenchyme from the molar tooth germs of wild type embryos rescued the development of the vestigial premolar rudiment. At the E12 stage some samples formed an extra tooth resembling the premolar rudiment formation in *Sostdc1*-deficient mouse in front of the first molar.

Sequential use of signaling pathways seems to take place in the enamel knot activation and both Wnt and Bmp pathways are shown to be involved. As Sostdc1 has been reported to interact with both canonical Wnt and BMP pathway, we wanted to find out the activator molecule responsible for the rudimental incisor formation. Our inhibition studies showed that the introduction of Wnt and BMP pathway antagonists, Dkk1 and Noggin, respectively, to the cultured *Sostdc1*-deficient teeth were both able to prevent the formation of the vestigial incisors. We observed that BMP4 releasing beads were able to accelerate the formation of the supernumerary tooth in *Sostdc1*-deficient tissue if placed by the lingual mesenchyme of the main incisor. As in the molar region the *Sostdc1* expression pattern correlated with *Bmp4* expression in the mesenchyme. The expression was, however, located in the buccal side in the molar region, whereas in the incisor region it was located at the lingual side. Compared to the *Sostdc1*, the *Bmp4* expression seemed to be in closer proximity to the lingual incisor epithelium at E13 stage. In addition the introduction of recombinant BMP4 protein in the culture medium of *Sostdc1*-deficient or trimmed wild type teeth from the Shh-GFPcre reporter mouse line, showed enlargement in the enamel knot region indicating the important role of BMP4 in the enamel knot activation. Similarly to our results, Murashima–Suginami et al. 2008 reported increased BMP4 activity from the *Sostdc1*-deficient mouse. The activation of canonical Wnt signaling with the GSK3beta inhibitor, BIO, failed to accelerate the rudimental incisor formation in the *Sostdc1*-deficient samples and seemed to disturbed the tooth morphogenesis.

The reduction of the surrounding mesenchyme from *Sostdc1*-deficient tooth was not expected to have any effect on the mutant phenotype since the rudimental extra incisors formed already *in vivo*. The trimming of the surrounding mesenchyme led, however, to the supernumerary *de novo* incisor formation from the lingual cervical loop epithelium of the main and the rudimental incisor. As they were forming from the epithelial region normally expressing *Sostdc1* this phenomena was not observed from the wild type samples. We confirmed this differential origin between rudimental and *de novo* incisors by using DiI- labeling. *De novo* molar formation was earlier published from the *Catenb*$^{\Delta ex3k14/+}$ mice after the forced activation of beta-catenin signaling in the epithelium (**Järvinen et al. 2006**). We investigated the formation of the incisors from the *Catenb*$^{\Delta ex3k14/+}$ mice *in vitro* and found a stronger supernumerary tooth formation from the lingual incisor epithelium of the previously formed tooth. This similarity between supernumerary tooth formations was suggestive that the epithelial *de novo* teeth formed in *Sostdc1*-deficient incisor *in vitro* was caused by activated canonical Wnt signaling in the lingual cervical loop epithelium.

Our work proposes a dual role for the tooth mesenchyme: during early development it is required for enamel knot activation, but later it appears to suppress tooth formation. This was observed from our *in vitro* culture studies, where the removal of surrounding mesenchyme advanced tooth formation in wild type, *Sostdc1*-deficient, and *Catenb*$^{\Delta ex3k14/+}$ samples. We showed Sostdc1 as one of these negative regulators in the mesenchyme, but we revealed the presence of another inhibitor, which prevented the *de novo* incisor formation from the lingual cervical loop of the *Sostdc1*-deficient incisors *in vivo*. The similarity between the *de novo* incisors in *Sostdc1*-deficient incisors with the supernumerary tooth formation in *Catenb*$^{\Delta ex3k14/+}$ mice suggested a role for the Sostdc1 as a Wnt antagonist in the epithelium. In the absence of epithelial *Sostdc1* the unknown mesenchymal antagonist may thus target the epithelial canonical Wnt pathway. Further studies are, however, required to reveal this other mesenchymal suppressor. Candidates to consider are mesenchymal Dkk1 or mesenchymal Wnt signaling since our results from BIO-treatment showed inhibition of the rudimental tooth development. In line with these results the negative regulator of canonical Wnt pathway, *Axin2*, showed upregulation in *Sostdc1*-deficient mesenchyme (**Ohazama et al. 2008**).

Instead of forming from a separate bud from the dental lamina, the extra incisor developed from the lingual epithelium of the main incisor resembling the formation of

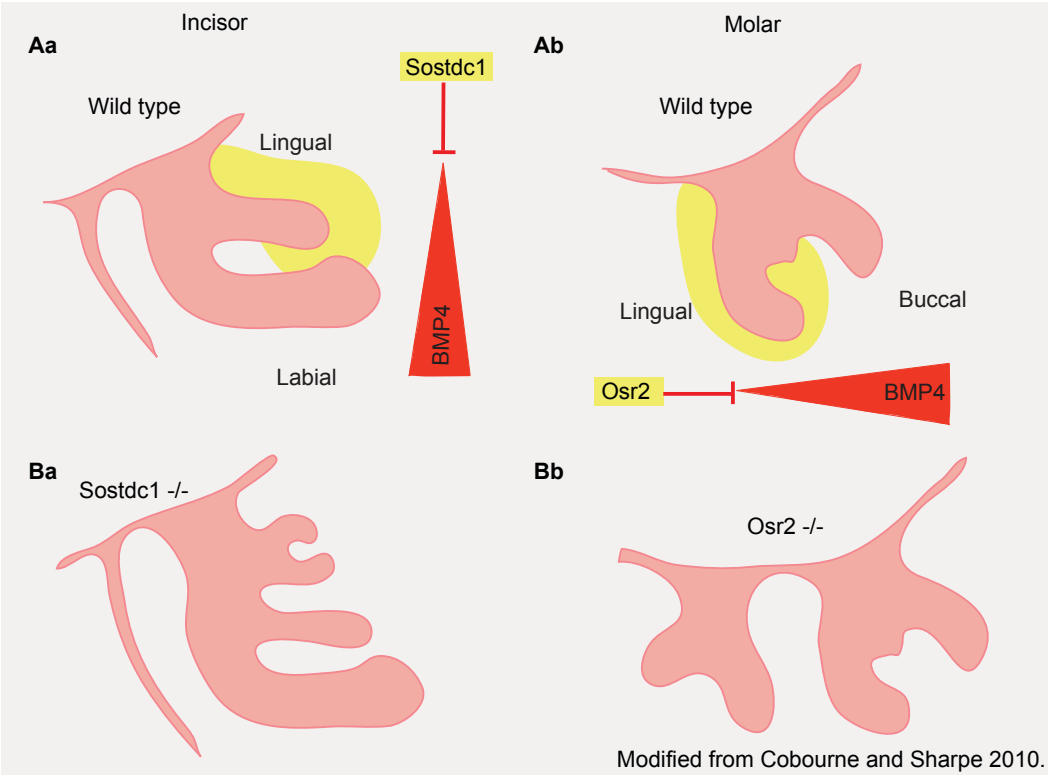

Figure 9. *Both Sostdc1 and Osr2 are expressed in the mesenchyme at the lingual side of the tooth (shown in yellow). They both antagonize the mesenchymal BMP4 signaling preventing the expansion of odontogenic field at the lingual side of the tooth (Aa, Ab). In the absence of Sostdc1, the supernumerary tooth develops at the lingual side of the main incisor (Ba) and in the absence of Osr2 supernumerary lingual molars form (Bb).*

replacement tooth (**Luckett, 1985; Järvinen et al 2009**). Rodent incisors are considered as first or second deciduous teeth (**Luckett, 1985; Meng et al. 2003; Asher et al. 2005**), whereas the permanent replacement teeth are thought to have disappeared during evolution. Our results propose a role for Sostdc1 in preventing the formation of these teeth in the modern mouse. In line with our results, Zhang et al. 2009 showed a development of lingual supernumerary molars in mice lacking the transcription factor *odd-skipped related-2* (Osr2). Like *Sostdc1* in the incisor region, the *Osr2* is expressed in lingual-to-buccal gradient in the molar region and has been shown to restrict mesenchymal odontogenic potential by inhibiting *Bmp4*. Our work, together with Zhang et al. 2009, showed suppressed tooth forming potential at the lingual side of mouse dentition, which may be suggestive of lost tooth replacement potential. In addition we propose a role for the Sostdc1 in the incisor region as a mesenchymal BMP4 and epithelial Wnt antagonist.

5.3. The fine-tuning of BMP and ActivinβA signaling regulates the size and number of incisors (III and unpublished results)

Next we wanted to investigate the molecular mechanism required for typical large rodent incisor formation. Peterkova et al. 1993 proposed a hypothesis that the modern mouse upper incisor may have formed from several distinct placodes, which all contribute to the formation of single maxillary incisor. They interpreted these several placodes to be potentially suggestive of the ancestral incisor formula.

The incisor develops from a considerably large placode compared to the molar. We visualized early incisor development (E11-E12) by using *Shh* as a placode marker, because it is expressed at E12 in the incisor placode, though it shifts later to the incisor enamel knot. In our previous work we showed that reduction in the surrounding mesenchyme at E13 stage lowers the expression of *Sostdc1* in the explants and leads to the lingual rudimental incisor formation *in vitro*. The same was observed at E11 and E12 except that earlier dissection into *in vitro* advanced the rudimental incisors formation. In addition, we observed that the main incisor was prone to dividing into two smaller teeth after trimming the incisor placodes at E12 stage. We used embryos from the Shh-GFPcre reporter mice and observed that reduction in the incisor surrounding mesenchyme at E11-E12 led to the formation of three small enamel knots. The two labial-most developed separately or fused later after growing in a close proximity. This is suggestive that the large mouse incisor is able to split into smaller teeth. The forming rudimental incisor is more slender than the main incisor, which could indicate the original size of the mouse incisor. The EDA null mutants have very fluctuating incisor phenotype varying from the absence of teeth to the extra incisors (unpublished observation). The *in vitro* culture experiments from these Tabby mice with the Shh-GFP reporter revealed that the main incisor forms, like in the wild type, from the two small placodes and they have the potential as well for the vestigial incisor formation.

In our previous works we showed that BMP4 has an important role in the enamel knot activation and its size regulation **(Kassai et al. 2005; Munne et al. 2009).** As the large size is typical for the incisor enamel knot, we investigated if the reduction in the mesenchymal BMP4 level could cause splitting of the main incisor into two smaller teeth. We treated E12 incisor explants with recombinant Noggin protein. The introduction of low concentration was observed to cause similar splitting of the main incisors as seen after trimming of the incisor surrounding mesenchyme at E12 stage. We showed earlier that

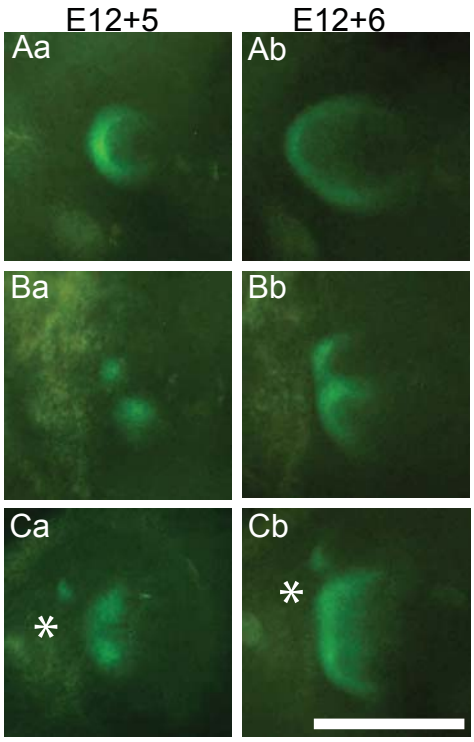

Figure 10. *Incisor explants from Tabby Shh-GFPcre reporter mice. In the control samples the Tabby incisor forms a long enamel knot, which is typical for the wild type mouse incisor (Aa, Ab). After trimming at E12 stage the tooth seems to form through a fusion of two smaller enamel knots (Ba, Bb). Also the rudimentary extra incisor is able to form to the Tabby incisor explants after the trimming of the surrounding mesenchyme at e12 stage (indicated by the asterisk) (Ca, Cb). Scale bar 0.5mm.*

mesenchymal BMP4 is crucial for the activation of rudimental incisor development. In line with this, the increased Noggin concentration was observed to prevent this rudimental incisor formation. Higher Noggin concentration caused the formation of only a single small incisor or none at all. This supported the role of mesenchymal BMP4 in the regulation of the enamel knot activity and size. *Bmp4* expression shifts in the mesenchyme at E12.5 stage. ActivinβA is another important TGF-β family member expressed simultaneously in the mesenchyme at this early stage. Both are needed for tooth development to proceed beyond the bud stage (**Ferguson et al. 1998; Matzuk et al. 1996; Chen et al. 1996**). We found that introduction of recombinant ActivinβA to culture medium at E10-E12 stage caused splitting of the main incisor similar to that seen after Noggin treatment or trimming. The tooth development was, however, slightly delayed when ActivinβA was present in the culture medium. This shows how the normal balance between mesenchymal ActivinβA and BMP4 is important for the main incisor integrity at E12 stage.

Next we looked more closely how this balance was normally regulated *in vivo*. We showed earlier that *Sostdc1* is an important inhibitor of mesenchymal BMP4. Follistatin, an antagonist of ActivinβA and BMP signaling, is as well active during tooth development. We observed that simultaneous deletion of these two antagonists,

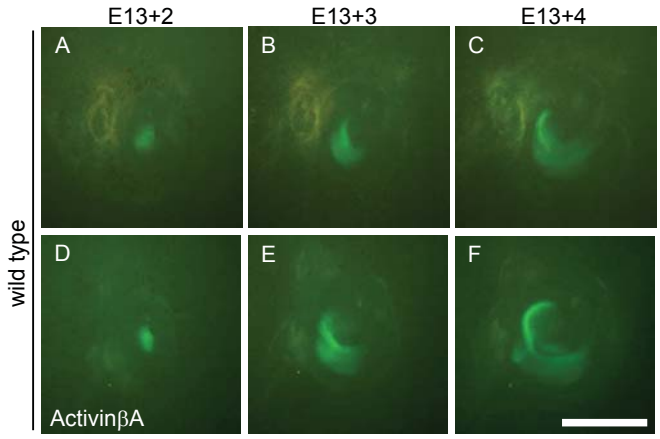

Figure 11. *The introduction of recombinant ActivinβA in the culture medium failed to affect the incisor integrity after E12 stage. A-C represents the control tooth and D-F the ActivinβA treated E13 wild type incisor with no evident change.*

Sostdc1 and *Follistatin*, caused a partially splitted incisor phenotype *in vivo* together with a rudimental extra incisor, which forms in the absence of *Sostdc1*. We looked at the expression of these two inhibitors during the early incisor development and observed their complimentary patterns at E12 and E13 stages. In addition to the lingual expression of mesenchymal *Sostdc1*, it was expressed in the epithelium, at the labial side of the tooth. This epithelial expression was complementary to *Follistatin*, which was expressed at the lingual side. We wanted to see the mechanism behind the formation of this partially split incisor in the *Sostdc1*/*Follistatin*-deficent mice by crossing them with Shh-GFPcre mice. This allowed the visualization of the enamel knot formation and revealed that the split in the main incisor in the double-mutant forms through simultaneous development of two small incisors, which will later partially fuse. Surprisingly in *Follistatin*-deficient mice two separate enamel knots were observed to form simultaneously in the main incisor region, but to fuse soon after to form only one normal looking mouse incisor. In *Sostdc1*-deficient mice the two enamel knots were observed as well, but the lingual one experienced a developmental delay and fused rapidly with the more advanced labial one. This proposes an interesting role for lingual *Follistatin* expression, which seems to prevent the simultaneous development of the two sides of the incisor bud. This was suggestive of the role of mesenchymal ActivinβA in maintaining epithelial proliferation, whereas the mesenchymal BMP4 is required for enamel knot activation.

A phenotype, similar to those found in *Sostdc1*/*Follistatin*-deficient or Noggin treated wild type incisors, was observed in K5-Smad7 mice (**Klopcic et al. 2007**). Smad7 is TGF-β/Activin induced TGF-β/Activin antagonist, which is also able to inhibit BMP pathway (**Nagarajan et al. 1999**; **Casellas and Brivanlou 1998**). Embryonic overexpression of the Smad7 under the K5 promotor caused defects in incisor patterning and enamel formation (**Klopcic et al. 2007**). The incisors from these mice indicated a problem with their incisor integrity by showing deep furrows that were fused from their bases. Interestingly the overexpression of Smad7 in the skin was observed to suppress the Wnt/β-catenin signaling (**Han et al. 2006**). During tooth formation high canonical Wnt signaling in the epithelium is found in the enamel knot. The overexpression of *Smad7* in tooth epithelium might suppress the WNT/β-catenin activity in the enamel knot and thus cause the perturbation in incisor integrity.

Our Noggin experiments at the E12 stage failed to show any change in tooth identity, although Tucker et al. 1998 showed that the introduction of exogenous recombinant Noggin at E10 jaws was able to change the incisor identity into molars. We repeated Noggin and ActivinβA treatment experiments at the E10 stage by introducing recombinant protein into the culturing medium. After the Noggin treatment the incisor region seemed to contain two small enamel knots, which led to the formation of two small incisors. This was indicated by the Shh-GFPcre reporter activity. The same was observed after ActivinβA treatment and additionally the whole dental epithelium was enlarged, which supports the hypothesis of mesenchymal ActivinβA as a stimulator of epithelial proliferation. Although the two small incisors start to develop separately they have a tendency to fuse while growing in a close proximity. This gave a multicusped appearance to these teeth, but their identity was confirmed to be incisors by kidney capsule culturing. Tucker et al. 1998 suggested that molar identity is dependent on *Barx1* expression, which is restricted to the molar region. Noggin treatment at E10 stage was shown, however, to allow the *Barx1* expression to spread into incisor region and therefore change the tooth identity. We investigated the effects of our Noggin experiments on *Barx1* expression pattern, but we did not observe any

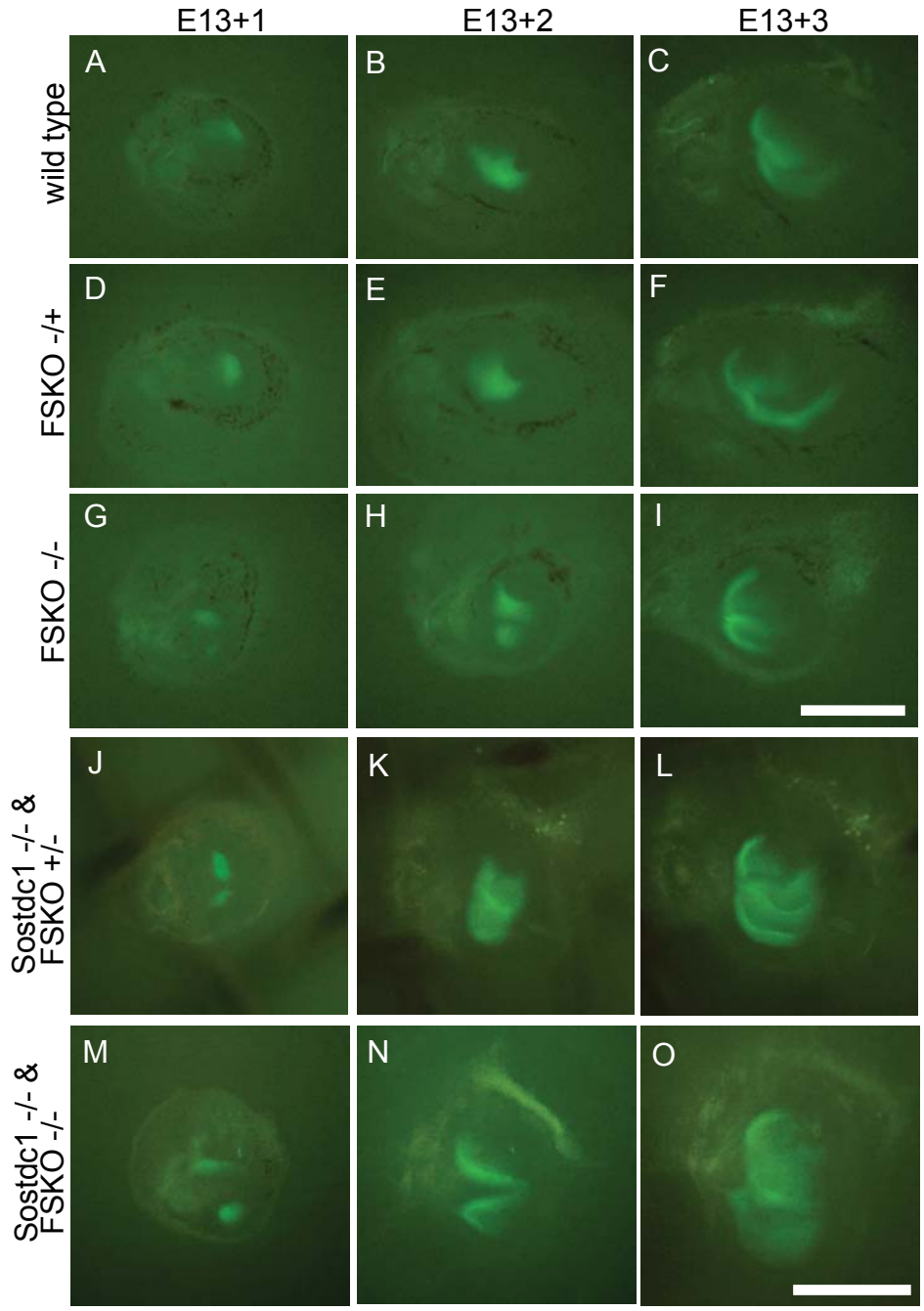

Figure 12. *The Follistatin appears to have a dose-dependent effect on the incisor phenotype. The Follistatin +/- incisors (D-F) are similar to the wild type incisors (A-C) at E13 stage. The absence of Follistatin alters the incisor development and instead of one long enamel knot, two small signaling centers appear simultaneously (G-I). They will, however, fuse later on and form normal sized incisor. The Follistatin +/- mice represent the similar incisor phenotype with the full Follistatin -/- mutants, if the Sostdc1 is simultaneously deleted (J-L). The Shh expression intensity is stronger in the absence of Sostdc1, which may reflect the increased signaling center activity. The complete absence of both Sostdc1 and Follistatin allows the development of two separate teeth (M-O). Scale bar 0.5mm.*

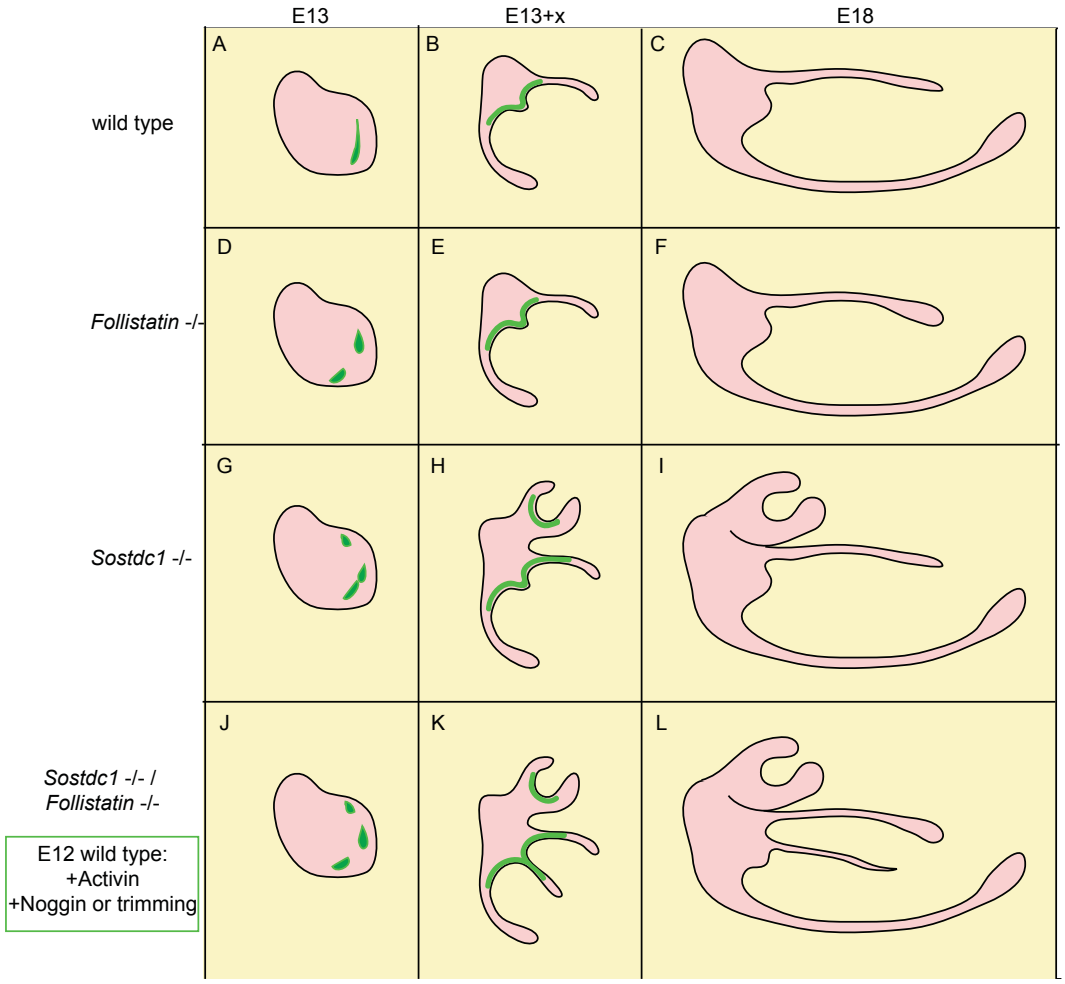

Figure 13. *Schematic drawing represents the early incisor development in different genetically altered mice compared to the wild type. The Follistatin-deficient incisor starts to form from two small simultaneously appearing enamel knots, which later fuse to form only one large mouse incisor (D-F). In the Sostdc1-deficient mice, these two small enamel knots form sequentially, which leads to their earlier fusion and thus to the normal incisor appearance. In addition the rudimentary extra incisor forms (G-I). In the absence of both Sostdc1 and Follistatin, the small enamel knots appear simultaneously and develop separately at first. Growing in the close proximity leads, however, to their fusion in their later development (J-L). In vitro culturing at E12 stage enables phenocopying the Sostdc1-deficiency to the wild type samples. Tinkering the balance between BMP and ActivinβA in this early developmental stage by inhibiting BMP with Noggin or increasing ActivinβA signaling with recombinant protein, perturbs the normal incisor development and leads to the splitting of the main incisor into two smaller teeth like seen in the Sostdc1/Follistatin-double mutants.*

difference at E10 or E12 stage. This is similar with the results from the mice with forced epithelial canonical Wnt activity (**Wang et al. 2009**). These mice form supernumerary uni- and multicusped teeth in the incisor region, but none of these teeth expressed *Barx1* in their dental mesenchyme. This is suggestive of *Barx1* having a role as a lateral mesenchymal marker instead of determining the tooth type. According to our preliminary studies, ActivinβA treatment seemed to increase *Barx1* expression in the molar region at E10 stage, but these results should be confirmed with a larger sample size.

In our previous work we showed that by culturing wild type incisors *in vitro*, explants are able to escape the effects of mesenchymal Sostdc1 (**Munne et al. 2009**). This is, however, not enough to cause a complete *Sostdc1*-deficiency *in vitro*, since wild type incisors at E13 have already some *Sostdc1* expression in their epithelium. Therefore, the *de novo* incisors that appeared to the full *Sostdc1*-deficient mice *in vitro*, were not observed from the wild type samples. We noticed, however, that if the wild type incisors are dissected at E12 and cultured *in vitro* with reduced amount of the surrounding mesenchyme or with mild Noggin concentration in the culture medium, it allows the wild type incisor cervical loop area to form *de novo*-incisors like those seen from the *Sostdc1*-deficient samples. This was observed only at E12 stage and after trimming the surrounding mesenchyme or Noggin treatment. The effects of trimming and Noggin treatment were both concluded to lower the effects of mesenchymal BMP4 and therefore lower the *Sostdc1* induction in the epithelium.

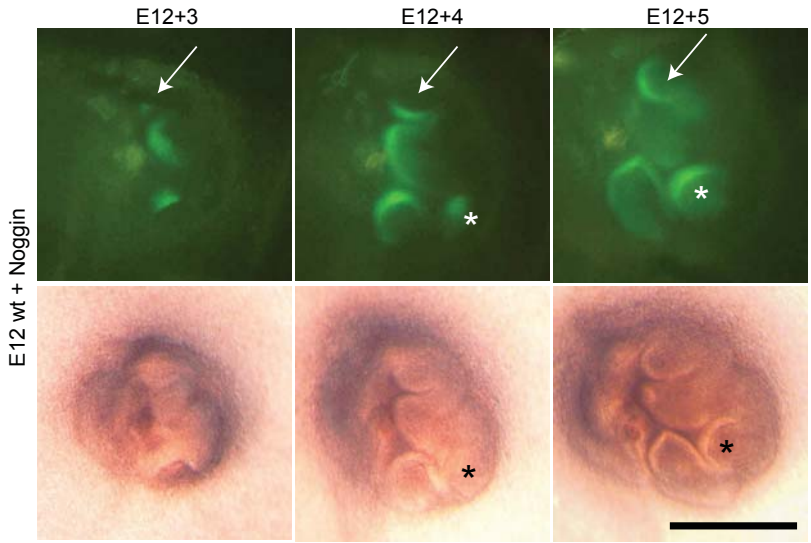

Figure 14. *In vitro culturing causes partial Sostdc1-deficiency in the wild type incisors and allows thus the rudimental extra incisor formation (indicated by the arrow). Normally the epithelial Sostdc1 expression starts at the cervical loop epithelium of the wild type incisors and prevents the de novo incisor formation observed from the full Sostdc1-deficient mice in vitro. Noggin treatment at E12 stage causes a slight inhibition in the mesenchymal BMP4 activity and thus the splitting in the main incisor into two smaller teeth. In addition de novo incisor formation was observed from the wild type samples after the Noggin treatment. This was suggestive that the slight BMP4 inhibition at E12 stage lowers the mesenchymal BMP4 activity and thus the Sostdc1 induction in the epithelium. An arrow indicates the rudimentary extra incisor and the asterisk the de novo tooth formation from the cervical loop epithelium. Scale bar 0.5mm.*

6. CONCLUDING REMARKS

"Existing diversity is a variation of the common theme" – Darwin

In order to understand the mechanisms, which lead to the phenotypic variation, we must first understand the genetic networks regulating organ shape, size, and number. This thesis work focused on the role of inhibitory molecules and especially on the role of signal pathway antagonists during the development of mouse dentition. In addition, this thesis suggests spatial differences in the TGF-β signaling and differential effects of BMP4 and ActivinβA on the tooth epithelium.

There seems to be different requirements for BMP4 in the incisor and molar region in the modern mouse. During initiation *Bmp4* is located in the incisor region, whereas *Fgf8* defines the molar region. BMP4 was shown to regulate the *Fgf8* expression in a dose-dependent manner (**Liu et al. 2005a**). A high dose of BMP4 signaling repressed *Fgf8*, but a low dose was required for *Fgf8* expression in the proximal region. In the absence of *Fgf8* the incisors were able to form although the molars failed to develop. FGF8 seems to be required for mesenchymal *Activin*β*A* expression (**Ferguson et al. 1998)**. This thesis work suggested opposite roles for mesenchymal BMP4 and ActivinβA during the incisor development. Reduction in BMP4 signaling in the early incisor development or increase in ActivinβA signaling causes the main incisor to divide into two smaller teeth. The reciprocal balance between these two TGF-β pathways appears to affect the ratio between epithelial proliferation and differentiation. The same was observed in limb development, where the ActivinβA was shown to promote epithelial survival by antagonizing the interdigital apoptosis caused by BMP4 (**Gañan et al. 1996; Merino et al. 1998**). The competition between these two TGF-β family members may be mediated by the limited pool of their common signal mediator, Smad4 (**Zimmerman et al. 1998)**. Exogenous Noggin shows increased epithelial cell survival in different contexts such as in the intestine epithelium, feather branching, and avian lip fusion (**Ashique et al. 2002; Yu et al. 2002; Haramis et al. 2004**). In feather formation, the antagonistic balance between BMP4 and Noggin is critical for branching. The BMP4 promotes rachis formation and barb fusion, and Noggin enhances rachis and barb branching (**Yu et al. 2002**). In tooth morphogenesis, high BMP4 signaling appears to lead to the fusion through the expansion of enamel knot area. Noggin and ActivinβA prevent this by increasing epithelial survival and thus allowing simultaneous formation of two small incisors. No change in the tooth identity was observed and the role of *Barx1* in this regulation was left open. Interestingly our preliminary whole mount *in situ* hybridization results showed that ActivinβA might be the upstream regulator of *Barx1* expression in the molar region (data not shown).

Regulation of BMP4 signaling seems to be crucial during morphogenesis. Rogulja and Irvine 2005 showed an interesting effect of the localized BMP signaling on patterning and growth. They showed that the juxtaposition of cells, which receive different levels of Dpp (BMP) signaling, is able to promote overall tissue proliferation. Either the activation or inhibition of BMP distinct from their surrounding cells caused a growth response in the adjacent cells. The uniform activation of DPP (BMP) in epithelial cells was show to inhibit proliferation. In line with these results, Kim et al. showed in 2007 that the co-localization of BMP4 together with Noggin beads in the mouse mandible increased the cell proliferation in the jaw. During the enamel knot formation *Bmp4* is expressed only in the underlying

mesenchyme, but it will later on appear in the enamel knot (**Jernvall et al. 1998**). Our *in vitro* culture studies with additional recombinant BMP4 in the medium were observed to enlarge the enamel knot area and accelerated molar crown differentiation in *Sostdc1*-deficient mice. The spatial delineation of BMP4 signaling appears to play an important role in morphogenesis by regulating spatial proliferation and differentiation. The same phenomenon was observed in the non-mammalian vertebrates, where homodont dentition appears to be the most common. Streelman and Albertson showed in 2006 that *Bmp4* expression seems to be associated with tooth site patterning in the early odontogenesis of cichlid fishes and may correlate with increased complexity of the dentition. These fishes have a relatively diverse dentition ranging from tightly-packed tricuspid teeth to widely-spaced unicusped teeth. Large *Bmp4* expressing domains correlated with fewer and larger unicusped teeth, whereas small *Bmp4* domains correlated with several small multicusped-teeth. This resembles the mouse dentition with large unicusped incisors and multicusped molars. It would be thus interesting to see if there is any correlation between ActivinβA and multicusped teeth in the cichlid fish dentition.

The large mouse incisor seems to be prone to disintegration after perturbation. Nakao et al. 2007 showed that the dissociated epithelial and mesenchymal single cells from E14.5 mouse incisors and molars formed multiple small teeth after re-association. The dissociation-re-association is likely to destroy the underlying morphogen gradients and homogenizes the molecular content. The multiple incisors may thus reflect tooth formation based solely on pattern formation dynamics. In normal morphogenesis spatial and temporal regulation of activator and inhibitor molecules is crucial. In limb development, the identity of different digits is based on the Noggin-sensitive BMP signaling gradient through the autopod (**Dahn and Fallon, 2000**). The labio-lingual asymmetry of the mouse incisor is known to follow from the inhibition of Activinβ and BMP pathways by Follistatin on the labial aspect of the incisor (**Wang et al. 2007**). This thesis work revealed the importance of spatial and temporal regulation of these two pathways in the early incisor morphogenesis of the mouse. Inhibitory molecules such as Sostdc1 and Follistatin are likely to modulate these signal pathways by creating spatial differences in proliferation and differentiation. When this spatial and temporal regulation is disturbed it allows the splitting of the placode and simultaneous development of two small incisors. This suggests that the mouse incisor region has the potential to form multiple small teeth. In addition to *Sostdc1/Follistatin*-deficient and K5-Smad7 mice, some other genetically altered mouse strains have a phenotype resembling this incomplete fusion of the incisors. Ohazama et al. 2010 showed that the hypomorphic mutant of *Lrp4* has grooved incisors. They interpreted these enamel-free zones as evolutionary conserved structures in vertebrates as they find these grooves from several other rodent species as well from the cichlid fishes. They did not, however, consider the possibility that instead of being cusps these grooves might represent incomplete fusion of splitted incisors. In the *Lrp4* hypomorphic mutant *Shh* expression in the enamel knot area was observed to decrease (**Ohazama et al. 2008**). This may have impaired the fusion of the enamel knots of two adjacent teeth forming the main incisor and thus led to a failure of complete fusion. This thesis work shows one example of how tooth number has decreased during evolution along with an increase in tooth complexity.

In the mouse the dentition is limited to a single row by restriction of odontogenic potential on the lingual side. The inhibitory molecules create disparity in the timing of development between adjacent forming structures. This is observed e.g. in the replacement

tooth formation, where the deciduous tooth appears to prevent the development of the permanent tooth (**Järvinen et al. 2008, 2009**). This thesis work showed dormant tooth forming potential on the lingual side of the mouse incisors. These epithelial cells are committed for the odontogenic program, but removed later by apoptosis. Their placement correlates with replacement tooth formation and they are thus suggestive of the dormant sequential tooth forming potential in the mouse. Their identity as a replacement tooth was not, however, confirmed. Interestingly, this potential is normally lost if the activation of odontogenic program does not take place before E14 stage. This inhibition seems to follow from the mesenchymal suppression of the enamel knot activator, BMP4. The activation of this lingual tooth formation took place after the removal of the mesenchymal inhibition. In some rare samples two extra incisors formed sequentially at the lingual side of the main incisor (data not shown). If this tooth formation represents suppressed tooth replacement potential, it raises a question, is the tooth replacement still a continuous process like seen in the lower vertebrates if the key inhibition is removed? Human Runx2 reduced function (cleidocranial dysplasia) causes the supernumerary teeth formation as a successional third dentition. This suggests that *Runx2* gene is involved in the inhibition of tooth replacement in humans by restricting it into two dentitions although there appears to be potential for continuous tooth formation. Activation of Wnt signaling in oral epithelium suggested that also mice have the potential for continuous tooth formation (**Järvinen et al. 2006**).Taken together, there seem to be at least two different levels of inhibition; the inhibition between two adjacent teeth to prevent their simultaneous formation and inhibition of sequential tooth development. Increased complexity in the dentition may be obtained with inhibitory molecules and more delicate regulation of activator inhibitor dynamics.

There appear to be many different mechanisms to regulate tooth number. Restricting the mesenchymal odontogenic potential from the buccal-lingual axis seems to be important regulator in the delineation of the dentition to a single row (**Zhang et al. 2009**). The inhibition of *Shh* signaling is commonly used to suppress the vestigial tooth buds during the development (**Ohazama et al. 2009**) and the restriction of epithelial canonical Wnt signaling seems to be important in controlling supernumerary *de novo* tooth formation (**Järvinen et al. 2006; Liu et al. 2008; Wang et al. 2009)**. This thesis work emphasizes the important role of a balance achieved by the fine-tuning of activators and inhibitors in order to create variation during a complex morphogenetic process.

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